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Inactivation and Elimination of Viruses during Preparation of Human Intravenous Immunoglobulin

Abstract

We report here the results of our evaluation of virus inactivation during the manufacturing steps of two intravenous immunoglobulin (IGIV) preparations. Virus inactivation and/or removal by processing steps, such as ethanol fractionation and polyethylene glycol precipitation, and deliberate virucidal steps, such as solvent/detergent treatment and pasteurization, were tested on a variety of human pathogenic and experimental model viruses, including human immunodeficiency, Hepatitis C, Mumps, Vaccinia, Chikungunya, Vesicular Stomatitis, Sindbis, and ECHO viruses. All viruses were successfully inactivated and/or eliminated by the processing steps studied. In some cases, however, multiple steps were required. We conclude that the incorporation of steps deliberately designed to inactivate or remove viruses during the production of IGIV provides an extra measure of viral safety.

Introduction

Transmission of a variety of human pathogenic viruses by blood [1–3] and blood products [3, 4–6] has led to the development of a number of procedures that inactivate viruses contained in these preparations. The risk of virus transmission is not equal for all plasma derivatives, and intravenous immune globulin (IGIV) products have consistently been associated with a low risk of virus transmission. Nevertheless, transmission of hepatitis B and non-A, non-B hepatitis has been reported, whether due to GMP noncompliance or failure of viral screening [7–15]. Although donor screening has apparently eliminated the risk of hepatitis B transmission, dependence on the screening procedure serves to highlight the need for safety methods that do not depend on identification of the virus. The experience with transmission of the human immunodeficiency virus (HIV) by blood products other than IGIV also mitigates in favor of stringent viral inactivation procedures that obviate possible dissemination of previously unrecognized viruses. Furthermore, the identification of hepatitis C virus (HCV), the major etiologic agent of non-A, non-B hepatitis, and the development of assays for the detection of HCV and antibodies directed at it [16, 17] have made possible a better understanding of the pathogenic potential of this virus.

Epidemiologic studies [18] reported by the US Centers for Disease Control and Prevention and the US Food and Drug Administration indicated that immunoglobulin produced by the Cohn-Oncley alcohol fractionation processes [19, 20] used in the United States carries no discernable risk of transmitting HIV. Viral inactivation/removal studies have supported this conclusion for HIV [21, 22]. In contrast, it appears that such methods may be inadequate for removing HCV; low levels of HCV RNA persisted after ethanol frac-

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tionation in a recent study [23]. Moreover, a plasma pool prepared from anti-HCV-negative donations (tested by the Ortho Diagnostics System), contained 1.6×10^3 polymerase chain reaction (PCR) units of HCV RNA/ml before processing. Based on virus removal during ethanol fractionation, the authors calculated that 'one might expect to find substantial amounts of HCV RNA in immunoglobulin prepared from unscreened pools and even in that from anti-HCV-screened pools (60 and 10 PCR units of HCV RNA/g IgG, respectively)' [23]. However, a positive PCR result does not necessarily imply infectivity.

A variety of techniques have been developed to inactivate potentially pathogenic human viruses in numerous therapeutic and experimental blood derivatives [24–29]. The incorporation of some of these steps into the preparation of IGIV has resulted in a new generation of products designed to provide a greater measure of viral safety, hence, IGIV preparations that incorporate a deliberate virucidal step have been referred to as 'third-generation immunoglobulins' [15]. The study presented here was designed to evaluate the efficacy of individual steps in two manufacturing processes used for the preparation of two third-generation IGIV preparations. A wide spectrum of viruses was studied, including both pathogenic human and experimental viruses; the viruses included both lipid-enveloped and non-lipid-enveloped viruses.

Materials and Methods

Plasma and Plasma Fractions

The plasma for these experiments was collected at plasmapheresis centers operated by Alpha Therapeutic Corporation (Los Angeles, Calif., USA). The samples for virus inactivation studies were prepared from source plasma during the regular manufacturing process. Plasma met the standards then in effect for source plasma (negative for hepatitis B surface antigen and anti-HIV-1-antibody, alanine aminotransferase level less than twice the upper limit of normal, but was not tested for antibody to HCV. Current standards also include screening for anti-HIV-2 and anti-HCV). All purification steps used in the manufacture of Venoglobulin®-S 5% Solution, Immune Globulin Intravenous (Human), Solvent Detergent Treated and Venoglobulin®-IH were reproduced in the Alpha Therapeutic Corporation and the Green Cross Corporation.

Venoglobulin-S was prepared from source plasma by cold ethanol fractionation according to the Cohn-Oncley procedures [19, 20]. Immunoglobulin G (IgG)-rich fraction II from the Cohn-Oncley process is further purified by polyethylene glycol (PEG) fractionation and anion exchange chromatography. The purified IgG is then treated with solvent detergent (SD, a mixture of tri-n-butyl phosphate [TNBP] and polysorbate 80, final concentrations of 0.3% and 1.0%, respectively) [24] as an additional virus inactivation step. After a second cation exchange chromatography step, the eluate is concentrated and *d*-sorbitol is added as a stabilizer. The purified IgG preparation undergoes final sterile filtration before aseptic filling as an aqueous solution into individual vials (fig. 1). Venoglobulin-S is currently licensed in the United States.

Venoglobulin-IH was prepared as previously described [30]. As shown in figure 2, the initial stages of purification of Venoglobulin-IH also rely on ethanol fractionation by the Cohn-Oncley procedures. Subsequent fractionation steps include PEG fractionation and ion exchange chromatography. The production of Venoglobulin-IH includes a pasteurization step (60°C, 10 h in aqueous solution) to inactivate viruses. Venoglobulin-IH is currently licensed in Japan and Europe. (In Europe, the product is marketed under the name Alphaglobin®.)

Virus Inactivation Studies

To examine the viral safety of IGIV prepared by the manufacturing processes used for Venoglobulin-S and Venoglobulin-IH, large amounts of human pathogenic and experimental viruses were deliberately added to material from different stages of the production process (spiking). The starting material for each step was spiked with an aliquot of virus from stock solution with the highest titer available. A control sample was taken after the addition of the virus to the protein solution. After subjecting the sample to the processing step, the remaining virus was determined according to established practice, as described below.

HIV Type 1 (HIV-1)

Human immunodeficiency virus type I (HTLV-IIIB) was prepared from the culture supernatants of MOLT-4/$HIV_{HTLV-IIIB}$ cells [31]. For the viral inactivation studies, the amount of HIV-1 was determined either as tissue culture infectious doses (TCID) or as plaque-forming units (PFU), as described below.

In some experiments (method 1), titers of HIV-1 were determined by culturing H-9 cells in the presence of 10-fold serial dilutions of test samples and controls. H-9 cells were seeded at a density of 10^5 cells per well. The samples were incubated for 10 days at 37°C with medium changes on days 3, 7, and 10. Samples of the culture supernatant were taken on day 10 and assayed for p24 antigen using an enzyme-linked immunosorbent assay (ELISA, Abbott Laboratories). The results for each sample were determined to be reactive or nonreactive and were expressed as $TCID_{50}$/ml. Virus for these studies was provided by Dr. S. Rasheed, University of Southern California, who also performed the HIV-1 assays on samples provided by the Research and Development Department.

In other experiments (method 2), titers of HIV-1 were determined by detection of infected target cells (H-9) exhibiting characteristic cytopathic effect (CPE) and by the release of HIV-1 into culture supernatants detected by reverse transcriptase assay or the expression of viral antigens detected by antigen capture assay. Samples were provided by the Research and Development Department and assays were performed by Advanced Bioscience Laboratories, Inc., Kensington, Md., USA.

PFU were determined by adding the HIV-spiked samples, before or after treatment, to MT-4 cells [32]. All experiments were carried out in triplicate. The limit of sensitivity of this method is 3.4 PFU/ml.

HIV Type 2 (HIV-2)

Titers of HIV-2 were determined by spiking test samples with HIV-2 prior to treatment, and assaying them after the viral inactivation procedure had been performed. Serial dilutions of test samples were added to HUT-78 target cells; after 3 weeks, cultures were evaluated for viable cell number, detection of characteristic CPE, and release of

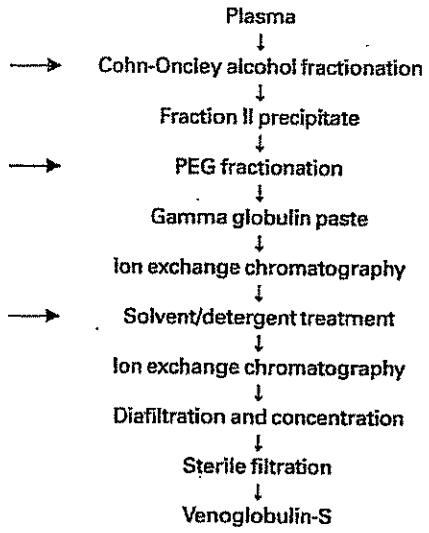

Fig. 1. Flow diagram of the manufacturing procedure used in the production of Venoglobulin-S.

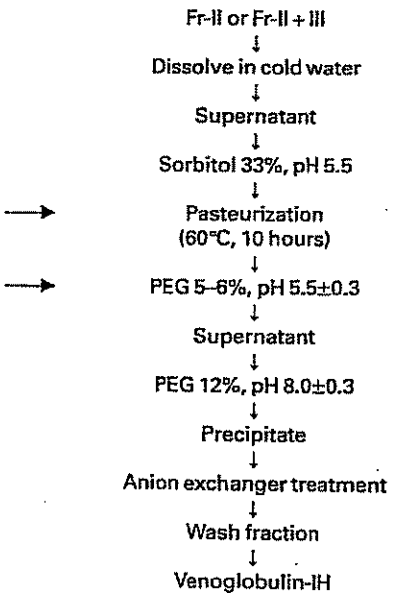

Fig. 2. Flow diagram of the manufacturing procedure used in the production of Venoglobulin-IH.

virus, as determined by reverse transcriptase assay. Samples were provided by the Research and Development Department and assays were performed by Advanced Bioscience Laboratories.

Hepatitis C Virus (HCV)

Quantitation of HCV RNA was accomplished by using a nested PCR at limiting dilutions as described by Yei et al. [23]. The titer of HCV is expressed as the highest dilution at which no viral RNA was detected by PCR. Anti-HCV-positive pool plasma was provided by Dr. Yei [23].

Vesicular Stomatitis Virus (VSV)

Vesicular stomatitis virus (strain Indiana) was propagated in Vero cells or FL cells. Infectivity was titrated by a plaque assay [33] expressed as PFU/ml. All assays were performed in duplicate. The limit of sensitivity of this system is 5 PFU/ml.

Sindbis Virus

Sindbis virus, which is a recognized model for HCV, was propagated in Vero cells or chicken embryo cells. Infectivity was titrated by a plaque assay [33] and expressed as PFU/ml. All assays were performed in duplicate. The limit of sensitivity of this system is 5 PFU/ml.

Chikungunya Virus (CHV)

Chikungunya virus (strain BaH306) was propagated in Vero cells. Infectivity was titrated by a plaque assay [33] and expressed as PFU/ml. All assays were performed in duplicate. The limit of sensitivity of this system is 5 PFU/ml.

Mumps Virus

Mumps virus (strain Urabe) was propagated in Vero cells. Infectivity was titrated by a plaque assay [33]. All assays were performed in duplicate. The limit of sensitivity of this system is 5 PFU/ml.

Vaccina Virus

Vaccina virus (Lister) was propagated in HeLa cells or FL cells. Infectivity was titrated by a plaque assay [33] and expressed as PFU/ml. All assays were performed in duplicate. The limit of sensitivity of this system is 5 PFU/ml.

ECHO Virus

ECHO virus (type 6) was grown in HeLa cells and its infectivity was titrated by cytopathic effect in HeLa cell cultures (expressed as $TCID_{50}$/ml). All assays were performed in duplicate.

Scale of the Experiments

4.5–9.0 ml of IgG sample, taken from an actual manufacturing run, and 0.5–1.0 ml virus stock solution were mixed and processed.

Results

Inactivation and Removal of HIV-1 by Initial Purification Steps

Plasma that was negative for anti-HIV-1 antibodies was used as the initial starting material for in vitro experiments to determine the amount of HIV-1 inactivated by steps in the Cohn-Oncley ethanol fractionation procedure and PEG fractionation. Samples representing each of the intermediate fractions in the Cohn-Oncley process (table 1) and a fraction II suspension in 4% PEG were spiked with HIV-1. The fractions were subjected to the conditions of the succeeding fractionation step. The amount of HIV-1 remaining in the resulting materials was tested using method 1 in the H-9 assay system, with quantitation of p24 antigen by ELISA.

We showed (table 2) that two logs of the virus were removed by partitioning into the fraction III precipitate. One additional log was removed by partitioning during the 4% PEG precipitation step. In addition, assuming that the level of HIV inactivation in each manufacturing step is independent of the starting titer at the beginning of each step, 10 additional logs of virus were inactivated during the Cohn-Oncley fractionation stage. Thus, the initial production steps studied reduced HIV-1, by inactivation and partitioning, by a total of 13 logs.

Inactivation and Removal of Human Pathogenic and Experimental Model Viruses by 4% PEG Precipitation

Starting material used to determine virus elimination by 4% PEG precipitation was prepared by suspending fraction II paste (1 kg) from an actual manufacturing run in cold water (10 l) and adding human albumin to a final concentration of 0.2%. Viral inactivation was tested by adding infectious human pathogenic virus and experimental marker viruses to an aliquot of this solution and measuring the amount of virus remaining after treatment with 4% PEG at 2°C for 1 h. As shown in table 3, 4% PEG precipitation eliminated 3.76 logs of Sindbis virus, 3.92 logs of VSV, 4.83 logs of Vaccinia virus, and 6 logs of PCR titer of HCV.

Inactivation and Removal of Human Pathogenic and Experimental Model Viruses by SD Treatment

The effectiveness of SD treatment in inactivating HIV-1 was determined. The two negative controls for this experiment were target cells incubated in medium containing 10% fetal calf serum plus antibiotics, and target cells incubated in unspiked Venoglobulin-S. The positive control was vi-

Table 1. Nominal conditions of the Cohn-Oncley steps studied

Step	Alcohol concentration, %	pH	Temperature °C	Setting time h
Fr I suspension	8	7.0±0.2	−2±1	0.5
Fr II + III suspension	20	6.9±0.1	−5.5±1.5	0.5
Fr II + IIIw suspension	20	7.2±0.1	−5±1	2
Fr III suspension	17	5.25±0.05	−6±2	10
Fr II suspension	25	7.25±0.05	−7±3	12

Fr = Fraction.

Table 2. Efficacy of the ethanol and polyethylene glycol (PEG) processing stages in removal or inactivation of HIV-1

Processing stage	Step	Log_{10} HIV-1 partitioned	Log_{10} HIV-1 inactivated	Total log_{10} HIV-1 reduction in step	Cumulative log_{10} HIV-1 reduction
Cohn-Oncley ethanol fractionation	Fr I supernatant	0	2	2	2
	Fr II and III precipitate	0	2	2	4
	Fr II and IIIw precipitate	0	2	2	6
	Fr III supernatant	2	1	3	9
	Fr II precipitate	0	2	2	11
PEG fractionation	Fr II suspension in 4% PEG	1	1	2	13

The starting material for each process step to be studied was spiked with an aliquot of HIV-1 with an initial titer of approximately 2.2×10^4 $TCID_{50}$/ml. Samples were then subjected to the conditions of the processing step and amount of HIV-1 partitioned and inactivated determined using H-9 target cells.

Fr = Fraction.

Table 3. Inactivation and removal of human pathogenic viruses and experimental marker viruses by 4% PEG fractionation (log_{10})

Virus	Added	Recovered		Eliminated	
		before PEG	after PEG	clearance	reduction
Sindbis	6.68	4.81	2.92	3.76	1.89
VSV	7.43	7.40	3.51	3.92	3.89
Vaccinia	7.75	7.38	2.92	4.83	4.46
HCV	6.0	4.0	0	6.0	4.0

Starting material prior to treatment with 4% PEG at 0–4°C was spiked with a sample of virus at the titer shown. Titers of Sindbis, Vesicular Stomatitis, and Vaccinia viruses are expressed as PFU/ml. The titer of HCV is expressed as the highest dilution at which no viral RNA was detected by PCR.

Table 4. Efficacy of the SD treatment in removing or inactivating infectious virus (log_{10})

Virus	Added	Recovered		Eliminated	
		before SD	after SD	clearance	reduction
Sindbis	6.68	6.60	<0.70	>5.90	>5.90
VSV	7.43	7.32	<1.86	>5.57	>5.46
Vaccinia	7.75	6.88	4.41	3.34	2.47
HCV	3.0	3.0	0	3.0	3.0
HIV-1	>11.5	>11.5	<1.5	>10	>10
HIV-2	6.5	5.3	<0.5	>6	>4.8

Starting material prior to solvent/detergent treatment was spiked with a sample of virus at the titer shown. Titers of Sindbis, Vesicular Stomatitis, and Vaccinia viruses are expressed as PFU/ml. The titer of HCV is expressed as the highest dilution at which no viral RNA was detected by PCR, and those of HIV-1 and HIV-2 as $TCID_{50}$/ml. Virus titers reported as '<' indicate complete inactivation within the limits of detection of the assay system used.

Table 5. Inactivation and partitioning (clearance) of human pathogenic viruses and experimental marker viruses during the manufacture of Venoglobulin-S

Processing step	Log_{10} virus eliminated					
	HIV-1	HIV-2	Sindbis	VSV	Vaccinia	HCV
Cohn-Oncley ethanol fractionation	11	ND	ND	ND	ND	ND
PEG fractionation	2	ND	3.8	3.9	4.5	6.0
SD treatment	>10	>6.0	>5.9	>5.5	3.3	3.0
Total	>23	>6.0	>9.7	>9.4	7.8	9.0

Starting material prior to SD treatment was spiked with a sample of virus at the highest virus concentration available. Titers of Sindbis, Vesicular Stomatitis, and Vaccinia viruses are expressed as PFU/ml. The titer of HCV is expressed as the highest dilution factor at which no viral RNA was detected by PCR, and those of HIV-1 and HIV-2 as $TCID_{50}$/ml.
ND = not determined.

rus-spiked Venoglobulin-S, untreated with SD, tested in target cells at dilutions up to 10^{-6}–10^{-5}. The inactivation tests were as follows: The SD mixture was added to the test samples (final concentration = 1% polysorbate 80 and 0.3% TNBP) taken from an actual production run. All tubes (controls and test samples) were incubated at 23°C for 30-, 60-, or 180-min inactivation periods. Following incubation, PEG (final 15%) was added to the samples to separate all viruses from the SD. The pellet was suspended in culture medium and tested for virus potency by method 2. Viral cultures were monitored weekly for 4 weeks for viable cell number, characteristic CPE, and expression of viral antigens as determined by antigen capture assay. Cultures expressing viral antigens were confirmed virus-positive by reverse transcriptase assay. A sample spiked to contain >11.5 log_{10} $TCID_{50}$ HIV-1 contained 1.5 log_{10} $TCID_{50}$ after incubation with SD for 30 min. No infectious virus was recovered from the samples inactivated for 60 or 180 min (table 4).

Similar studies revealed that SD treatment at 27°C for 30 or 180 min completely inactivated HIV-2 and HCV as well as the experimental marker viruses, Sindbis and VSV, to within the limits of sensitivity of the assay system (table 4). SD treatment also eliminated 2.5 logs of vaccinia virus.

Table 6. Inactivation of human pathogenic viruses and experimental marker viruses by pasteurization (log_{10})

Virus	Added	Recovered		Eliminated	
		before PEG	after PEG	clearance	reduction
Sindbis	7.58	6.83	<1.7	>5.8	>5.1
VSV	7.45	7.15	<1.7	>5.7	>5.4
Vaccinia	5.92	5.85	<1.7	>4.2	>4.1
HIV-1	5.81	5.62	<1.0	>4.8	>4.6
CHV	6.98	5.95	<1.7	>5.2	>4.2
Mumps	6.46	4.61	<1.7	>4.7	>2.9
ECHO	7.30	5.51	<1.7	>5.6	>3.8
HCV	3.00	3.00	1.0	2.0	2.0

Titers of all viruses are expressed as PFU/ml with the exception of ECHO virus (log_{10} TCID/ml) and HCV (highest dilution factor at which no viral RNA was detected by PCR). Virus titers reported as '<' indicate complete inactivation within the limits of detection of the assay system used.

Table 7. Inactivation and removal of human pathogenic viruses and experimental marker viruses by 6% PEG fractionation (log_{10})

Virus	Added	Recovered		Eliminated	
		before PEG	after PEG	clearance	reduction
Sindbis	7.85	7.16	<0.7	>7.1	>6.4
VSV	7.68	7.40	<0.7	>6.9	>6.7
Vaccinia	7.54	6.78	<0.7	>6.8	>6.0
HIV-1	4.64	1.95	<0.5	>4.1	>1.4
ECHO	8.00	6.70	<1.0	>7.0	>5.7
HCV	3.0	3.0	0	3.0	3.0

Starting material prior to treatment was spiked with a sample of virus at the titer shown; samples were then treated with 6% PEG, with the exception of the HIV sample, which was treated with 4% PEG. Titers of Sindbis, Vesicular Stomatitis, Vaccinia viruses and HIV are expressed as PFU/ml. The titer of HCV is expressed as the highest dilution factor at which no viral RNA was detected by PCR, and that of ECHO virus as $TCID_{50}$/ml. Virus titers reported as '<' indicate complete inactivation within the limits of detection of the assay system used.

Cumulative Inactivation and Removal of Viruses during the Manufacture of Venoglobulin-S

Table 5 summarizes the inactivation and removal of viruses by the steps used in the manufacture of Venoglobulin-S. Cumulatively, these procedures eliminated >23 logs of HIV-1; >6 logs of HIV-2; >9.7 logs of Sindbis virus; >9.4 logs of VSV; 7.8 logs of Vaccinia; and 9.0 logs of PCR titer of HCV.

Inactivation of Human Pathogenic and Experimental Marker Viruses by Pasteurization

Inactivation of Sindbis virus, VSV, Vaccinia virus, HIV, CHV, Mumps virus, ECHO virus, and HCV by heating in the presence of 33% (weight/weight) sorbitol is shown in table 6. Fraction II + III paste was suspended in cold water; the pH was adjusted to 5.5, and the material was centrifuged to produce a clear supernatant. Sorbitol was added to the IgG-containing supernatant, and the pH was adjusted to 5.5 if necessary. Samples were spiked with virus stock solution prior to heating (60°C, 1 h), and the amount of infectious virus remaining after pasteurization was determined. All of the viruses were completely inactivated within the limits of detection of the assay systems used, with the exception of HCV, the PCR titer of which was reduced by 2 logs by the procedure.

Inactivation and Removal of Human Pathogenic and Experimental Marker Viruses by 6% PEG Precipitation

Elimination of infectious Sindbis virus, VSV, Vaccinia virus, HIV-1, ECHO virus, and HCV by PEG precipitation is shown in table 7. IgG solution containing 33% sorbitol was heated at 60°C for 10 h (pasteurization) and diluted with cold distilled water three times to reduce the sorbitol concentration. Virus stock solution was added to the diluted pasteurized IgG solution, and PEG was added. Samples containing 6% PEG (4% PEG in the case of the HIV-1-spiked samples) were held at 0–4°C for 1 h and centrifuged to remove aggregated IgG, other protein impurities, and viruses. The supernatant, containing monomeric IgG, was tested for remaining virus. After treatment with PEG, infectious virus was undetectable within the limits of sensitivity of the assay systems used. PEG fractionation used in the preparation of Venoglobulin-IH eliminated >7.1 logs of Sindbis virus; >6.9 logs of VSV; >6.8 logs of Vaccinia virus; >4.1 logs of HIV; >7 logs of ECHO virus; and eliminated all of the PCR titer of 3 logs of HCV added to the test sample.

Cumulative Inactivation and Removal of Viruses during the Manufacture of Venoglobulin-IH

Table 8 summarizes the inactivation and removal of viruses by the steps used in the manufacture of Venoglobulin-IH. Cumulatively, the amount of elimination of viruses (giv-

Table 8. Cumulative inactivation and removal (clearance) of viruses during the manufacture of Venoglobulin-IH

Processing step	log_{10} virus removed/inactivated							
	HIV	CHV	Mumps	Sindbis	VSV	Vaccinia	ECHO	HCV
Cohn-Oncley ethanol fractionation	11	ND	ND	ND	ND	ND	ND	ND
Pasteurization	>4.8	>5.2	>4.7	>5.8	>5.7	>4.2	>5.6	2.0
PEG fractionation[1]	>4.1	ND	ND	>7.1	>6.9	>6.8	>7.0	3.0
Total	>19.9	>5.2	>4.7	>12.9	>12.6	>11.0	>12.6	5.0

Titers of Sindbis, Vesicular Stomatitis and Vaccinia viruses, and of HIV are expressed as PFU/ml. The titer of HCV is expressed as the highest dilution factor at which no viral RNA was detected by PCR, and that of ECHO virus as $TCID_{50}$/ml.

ND = Not determined.

[1] PEG treatment was 6%, with the exception of HIV-1 (4%).

en in log_{10}) by these procedures was >19.9 for HIV-1; >5.2 for CHV; >4.7 for Mumps virus; >12.9 for Sindbis; >12.6 for VSV; >11 for Vaccinia; >12.6 for ECHO virus; and 5 logs of PCR titer for HCV.

Discussion

The excellent safety record with respect to viral transmission by IGIV products is attributed to a combination of several factors: (1) reduction of infectious viruses in plasma by identification of infected donors and elimination of this plasma from plasma used for the purification of IGIV, (2) blocking of virus infectivity by antibodies in the IGIV preparation, and (3) viral elimination and inactivation by methods used during the manufacture of IGIV. The first two factors may be in a precarious state of balance, since, as better methods are developed to detect infected donors, potentially advantageous blocking antibodies may be eliminated from the donor pool. The ethanol fractionation procedure used to prepare IGIV provides one means of viral inactivation, and other steps in the different purification processes may also fortuitously include some measure of inactivation. Nevertheless, these steps should be evaluated for their ability to remove or inactivate viruses. Thus, as discussed below, it is suggested that a fifth level of safety be incorporated into IGIV products, namely, that deliberate viral inactivation steps be included in their production. This approach provides the highest level of confidence that IGIV products are free of infectious virus.

Epidemiologic data reported by the Centers for Disease Prevention and Control support the general experience that IGIV products are remarkably safe from viral transmission compared with other, more labile, plasma derivatives. In 1985, Piszkiewicz et al., [21] reported the results of in vitro experiments that showed that the HIV-1 was effectively inactivated by the fractionation steps of the Cohn Oncley ethanol fractionation procedure used in the preparation of IGIV. The authors concluded that the 'steps in large-scale plasma fractionation, especially ethanol at –5°C and low pH, reduce the risk of plasma products being infectious should the source material be contaminated by HTVL-III/LAV [HIV-1]'. In 1986, Wells et al. [22] reported the results of similar experiments, and determined that ethanol fractionation of human plasma was able to reduce infectious HIV by 15 log_{10}, based on the assumption that each step was independent from other steps. In similar experiments reported here, the Cohn-Oncley cold ethanol fractionation steps cumulatively eliminated at least 11 logs of HIV-1, which is consistent with the results of Wells et al.

In contrast to results with HIV, both in vitro viral inactivation studies [23] and sporadic reports of hepatitis C transmission by IGIV products [10–15] suggest the need to incorporate specific viral inactivation steps (in addition to ethanol fractionation) in the production of IGIV in order to obviate the possibility of hepatitis C transmission.

PEG fractionation is an effective means of removing aggregated immunoglobulin from IGIV preparations. It is also commonly used as a concentration technique for viruses. We have previously shown [32] that precipitation with 5% PEG at low ionic strength was effective for the elimination of $>10^4$ PFU of HIV-1 and $>10^5$ units of other viruses, including Hepatitis B virus, Vesicular Stomatitis virus, and Sindbis virus. Precipitation with 4% PEG, which is included in the manufacturing process of both Venoglobulin-S and Venoglobulin-IH, was shown to eliminate 3.76 logs of Sind-

bis virus, 3.92 logs of VSV, 4.83 logs of Vaccinia virus, and 6 logs of PCR titer of HCV. In addition, 2 logs of HIV-1 TCID and >4.1 logs of HIV-1 PFU were eliminated by 4% PEG. These results are consistent with our previous studies.

SD treatment is a process that was devised as a specific method to inactivate lipid-enveloped viruses [24, 26–29]. The results of this study show that SD treatment for either 30 min or 180 min completely inactivates HIV-1, HIV-2, Sindbis virus, VSV, and HCV within the limits of sensitivity of the assay systems used for virus detection; this means that the treatment is capable of eliminating >10 logs of HIV-1; >6 logs of HIV-2; 5.9 logs of Sindbis virus; 5.5 logs of VSV, and 3 logs of PCR titer of HCV. The length of the SD manufacturing step used to produce Venoglobulin-S is 6 h, which is 12 times longer than the minimum time (30 min) necessary for complete inactivation of the viruses tested. Unlike the other lipid-enveloped viruses tested in this system, vaccinia virus was not completely inactivated by the SD treatment step.

Like SD treatment, pasteurization was developed to inactivate viruses contained in therapeutic plasma derivatives. Heat treatment has the potential to eliminate both lipid-enveloped and non-lipid-enveloped viruses, but unless a stabilizer such as sorbitol is added, immunoglobulin is easily denatured under heating conditions that are virucidal. We have previously shown that heating for 10 h at 60°C in solution containing 33% sorbitol at low ionic strength is an effective means of inactivating HIV, Mumps, Vaccinia, and four other viruses while retaining the physicochemical and biological properties of unheated IgG preparations [30].

In the studies reported here, infectious viruses, including HIV, CHV, VSV, Mumps, Vaccinia, ECHO, and HCV, were completely inactivated when heated at 60°C for 1 h in a 5% IgG solution with 33% sorbitol. These results are consistent with those of other investigators that show pasteurization to be an effective means of providing an extra measure of viral safety in plasma derivatives [34, 35]. The actual process of pasteurization used in the manufacture of Venoglobulin-IH is 10 h at 60°C, which is 10 times longer than the minimum time needed for complete viral inactivation (1 h), thus the amount of virus inactivated during the manufacturing process may actually be higher.

In experiments designed to evaluate the viral safety conferred by ethanol fractionation, Yei and Tankersley [23] concluded that 'in spite of an excellent safety record, the detection of HCV RNA in immune globulin, coupled with sporadic reports of non-A, non-B hepatitis transmission by intravenous immune globulin, suggests that virus-inactivation and/or -removal steps should be considered for immune globulin products'. That study highlights the need for understanding the potential for viral inactivation at various stages in the production procedure as well as the cumulative viral inactivation that results during the actual preparation of blood derivatives subjected to procedures that inactivate or partition viruses.

Conclusion

The experiments reported here may underestimate the amount of viruses actually eliminated during the manufacturing processes of Venoglobulin-S and Venoglobulin-IH, which contain additional steps and/or longer inactivation times. Therefore, for Venoglobulin-S the conservative estimate for the accumulated amount of removal/inactivation is >23 logs HIV-1 (three steps tested); >6 logs HIV-2 (one step tested); >9.7 logs Sindbis virus (two steps tested); 7.0 logs Vaccinia virus (two steps tested); and 9.0 logs of PCR titer of HCV (two steps tested). The conservative estimate for the accumulated viral inactivation for the steps used in the manufacture of Venoglobulin-IH is >19 logs HIV (three steps tested); >5.2 logs CHV (one step tested); >4.7 logs Mumps virus (one step tested); >12 logs Sindbis virus (two steps tested); >12 logs VSV (two steps tested); >11 logs Vaccinia virus (two steps tested); >12 logs ECHO virus (two steps tested); and 5 logs of PCR titer of HCV (two steps tested).

The experiments reported here show that the combination of different methods that have different mechanisms for inactivating or eliminating viruses from immunoglobulin products provides viral inactivation that is superior to that found in any single inactivation step. We conclude that, although the fractionation steps designed for the purification of IgG in IGIV for therapeutic application do eliminate infectious virus, an extra measure of viral safety can be achieved by incorporating steps deliberately designed to inactivate or remove viruses during the production of IGIV.

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A Seral Epidemiological Study of HIV Transmitted through Human Seral γ-Globulin Preparations

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Li J (Jining Hygiene and Antiepidemic Station, Shandong, People's Republic of China), Jiang DH, Wang LF, Zeng Y, Li D, Li GX, Liu YY, Shao YM, Zhu ZZ, Kong J, Feng XX, Jing SN, Wang J and Liu JX. A seral epidemiological study of HIV transmitted through human seral γ-globulin preparations. *International Journal of Epidemiology* 1990; 19: 1057–1060.
In order to study the potential risk of transferring HIV through human seral γ-globulin preparations (immunoglobulin), indirect immunofluorescent antibody test (IFA) and Western Blot (WB) assay were applied to 343 random samples (sera) with previous injection of imported human seral γ-globulins (Ig) positive for Human Immunodeficiency Virus (HIV) antibodies between 1981–1987 for the detection of HIV antibodies. All results were negative and tests on all 23 controls who had previously received Ig made in China also gave negative results. However all 12 batches of imported Ig collected from the above-mentioned users, were positive for HIV antibodies when tested by WB and IFA. This study shows that under normal conditions, human seral γ-globulin does not transmit HIV.

Since 1981, with the discovery of AIDS and its growing incidence, people have become more and more worried about the safety of blood products. There have been reports of the detection of HIV antibodies in human seral γ-globulin (Ig) that is commercially available,[1–6] thus raising the fear of transferring live HIV into blood through injection of immunoglobulins. In order to study the potential risk of transferring HIV through immunoglobulins, 366 random samples were drawn from people who had received γ-globulin to detect the level of HIV antibodies. The results are as follows.

MATERIALS AND METHODS

Sera

From August 1987 to March 1988, sterile collections of sera were conducted by randomization[7] from 343 individuals who had received imported human γ-globulins between 1981 and 1987, and from 23 controls who had previously received injections of γ-globulins in China. All the sera were stored at −20°C.

Human Seral γ-globulin (Ig)

Fourteen batches of imported human γ-globulins and five batches of globulins made in China were collected from the above mentioned 366 individuals and kept at 0°C.

Testing Methods

Indirect Immunofluorescent Antibody (IFA) and Western Blot (WB) techniques were used to detect HIV antibodies from the sera and immunoglobulins.
(i) Indirect immunofluorescent Antibody Test (IFA).[27] HIV antigens—H9 cells infected by HIV were used in preparing the IFA slides at the Virus Institute of the Chinese Academy of Preventive Medicine.
(ii) Western Blot technique (WB).[27] Compared with standard positive sera, those with viral protein patterns—p24 and gp41 are regarded as positive.

Criteria Used to Determine Positivity

(i) IFA and WB both positive are regarded as positive.
(ii) Only WB positive is regarded as positive.
(iii) Only IFA positive is regarded as negative.

RESULTS

Seral Test

In this survey, sera from 366 Ig users were tested, 343

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of which had used imported Ig and 23 with Ig made in this country P R China. All the sera were confirmed to be HIV negative after being tested with IFA and WB techniques by the Virus Institute of the Chineses Academy of Preventive Medicine.

Results of Test with Immunoglobulins

Fourteen batches of imported Ig and five batches made in this country were collected from the above-mentioned users and tested with IFA technique and WB Assay by the Virus Institute of the Chinese Academy of Preventive Medicine. The 12 batches of imported Ig were found to be positive for HIV antibodies while all five batches made in China were negative (Table 1).

Physical Examination

In this survey, a physical check on all the 366 people who had received Ig injections did not reveal any signs or symptoms of AIDS.

Age Constitution

Of the 366 individuals, the youngest was two years old and the oldest 72 years old. The age distribution of the examined population was as follows:

under 10 years of age—61.2% (224)
10–19 year olds—14.21% (52)
20–29 year olds—5.46% (20)
30–39 year olds—9.56% (35)
40–49 year olds—5.74% (21)
50 and over—3.83% (14)

Sex

Among those previously receiving imported Ig 172 were males and 171 females; among those receiving Ig made in China 11 were males and 12 females. Origin of Ig and the number of people who received them is shown in Table 2. Among the 366 individuals who had had Ig injections, there were 202 people with Ig injection made in Spain, 84 people with Ig injections made in Austria, 27 people who received Ig injections made in the USA, 23 controls who received Ig injections made in CHINA, 11 people who received Ig injections made in Japan and 19 people who received Ig injections made in other countries, including GDR, Italy and Singapore.

Dosage of Injection and Time Distribution (Table 3)

In Table 3, it is shown that the interval between the last γ-globulin injection and the time of serum collection is between one and seven years (1981–1988).

Of the subjects and controls, there were four people who had an Ig injection in 1981. The dosage was 320 mg/person in one injection.

In 1982, there were 25 people who had an Ig injection, 13 of whom received 320 mg/person in one dose, eight of them had 320 mg/day for two consecutive days, and four of them had the same dose for three consecutive days.

In 1983, there were 72 people who had an Ig injection: eight of them received 160 mg in one injection, 41 of them had 320 mg in one injection and the other 23 people had 640 mg/day for between one and five consecutive days.

In 1984, there were 76 people who received Ig injection: four of them had 160 mg each and 50 had 320 mg

TABLE 1 *Test for HIV antibodies in the 14 batches of imported Ig and 5 batches of Ig made in China*

Sample name	Manufactured	Brand	Batch	IFA	Western Blot
Human serum γ-globulin	Spain	Hubber	v-5	+	+ (p24+ gp41+)
	Spain	Glogama	x17	+	+ (p24+ gp41+)
	Spain	Do-globin	x046	+	+ (p24+ gp41+)
	Spain	Figlubin	T-003-E	+	+ (p24+ gp41+)
	Spain	Figlubin	T-012-E	+	+ (p24+ gp41+)
	Spain	Figlubin	T-019-E	+	− (p24− gp41−)
	Spain	Grifols	V06	+	+ (p24+ gp41+)
	Spain	Grifols	V11	+	+ (p24+ gp41+)
	Spain	Grifols	V25	+	+ (p24+ gp41+)
	Spain	Grifols	V36	+	− (p24− gp41−)
	Spain	Grifols	V37	+	+ (p24+ gp41+)
	Austria	—	622624	+	+ (p24+ gp41+)
	Austria	—	620175	+	+ (p24+ gp41+)
	Austria	—	621124	+	+ (p24+ gp41+)
	China	—	790614	−	− (p24− gp41−)
	China	—	840326	−	− (p24− gp41−)
	China	—	850620	−	− (p24− gp41−)
	China	—	870324	−	− (p24− gp41−)
	China	—	830416	−	− (p24− gp41−)
Total	3 countries		19 batches	14+/5−	12+/7−

TABLE 2 *Country of orig*

Country	Brand	Ba
Spain	Grifols	V1
	Hubber	V5
	Figlubin	T-0
	Figlubin	T-3
	Glogama	T34
	Do-globin	X1
Austria		200
US		
Japan		
GDR		
Italy		
Singapore		
Other		
China		
Total		

in one injection; 22 p
between one and three

In 1985, there were
injection: six of them ha
had 320 mg in one inject
for between one and fiv

In 1986, there were 61
three of them had 160 n
320 mg in one injectio
640 mg/day for betwee
days. In 1987, 25 peopl
320 mg in one injection
640 mg/day for betwee
days (Table 3).

TA

Dosage of injection (mg)	1981
160	
320	4
640	
960	
1280	
1600	
1920	
2240	
2560	
5120	
6400	
Total (Person)	4

DISCUSSION

One of the modes for A

TABLE 2 *Country of origin of each Ig and people receiving each*

Country	Brand	Batch	People receiving Ig (person)
Spain	Grifols	V11,V25,V06,V36,V37	45
	Hubber	V5	34
	Figlubin	T-003E,T-019E,T-012E	41
	Figlubin	T-311	39
	Giogama	T34,X17	43
	Do-globin	X137,T-102E,X-046	45
Austria		2007082,621324,622624, 100883,620175,	39
US			27
Japan			11
GDR			1
Italy			1
Singapore			1
Other			16
China			23
Total			366

in one injection; 22 people had 640 mg each for between one and three consecutive days.

In 1985, there were 103 people who received Ig injection: six of them had 160 mg each and the other 73 had 320 mg in one injection; 24 people had 640 mg/day for between one and five consecutive days.

In 1986, there were 61 people who had Ig injection: three of them had 160 mg/person and 42 of them had 320 mg in one injection; the other 16 people had 640 mg/day for between one and nine consecutive days. In 1987, 25 people injected Ig, 13 of them had 320 mg in one injection and the other 12 people had 640 mg/day for between one and three consecutive days (Table 3).

DISCUSSION

One of the modes for AIDS transmission is through blood transfusion and/or use of blood products. The average incubation period is five years or longer,[8] with an extremely high mortality rate. With the acquisition of HIV infection and production of antibodies an individual can either become severely ill or remain an asymptomatic carrier. Data[1-6] have confirmed the presence of HIV antibodies in immunoglobulin products, indicating the risk of receiving live virus through injections. However, it is questionable whether man can become seral HIV antibody positive with an injection of Ig. If this is possible how long does the positive reaction last? Theoretically, assuming from the half-life of human Ig, the persistence of HIV antibodies within the body depends on the dosage of the injection, idiosyncrasy of the recipients, construction of HIV antibodies and other possible factors. Yet the above-mentioned phenomenon has not been reported,[1,9-12] indicating that it is rare. However, it is possible that current laboratory techniques may not be able to detect trace amounts of HIV antibodies[13] in serum samples. Hein et al.[14] injected HIV antibody positive Ig intravenously into six volunteers in 1983; the dosage was 250–500 mg/kg/day for five consecutive days. After two years the sera of these volunteers were all negative for HIV antibodies when tested by ELISA techniques. No changes of T-cells were noted. Drotman[15] has made clinical observations of 11 hypo-γ-globulinaemic cases who had received large doses of Ig intravenously and did not find proof of seral positivity after injection with Ig products containing HIV antibodies[15]. Gocke et al applied ELISA techniques to examine 17 batches of different Ig made by different manufacturers and found 16 batches positive for HIV antibodies. Chinese researchers have been using ELISA, IFA and WB techniques to test human γ-globulins from countries with high AIDS incidence for the presence of HIV antibodies. The results were positive.[4-6] In the survey,

TABLE 3 *Relationship between dosages and time of injections with the 366 individuals*

Dosage of injection (mg)	Time of injection (year)							Time of serum collection 1988	
	1981	1982	1983	1984	1985	1986	1987	Total (Person)	Percentage
160			8	4	6	3		21	5.74
320	4	13	41	50	73	42	13	236	64.48
640		8	13	16	7	7	4	55	15.02
960		4	7	5	8	2	7	33	9.02
1280			1	1	5	2	1	10	2.73
1600			1		2			3	0.82
1920			1		1	1		3	0.82
2240						1		1	0.27
2560						1		1	0.27
5120						1		1	0.27
6400					1	1		2	0.56
Total (Person)	4	25	72	76	103	61	25	366	100%

individuals were chosen who had received immunoglobulins positive for HIV antibodies imported from high risk countries between 1981–1987. Sera from 343 such samples and from 23 recipients of Ig made in China (control group) were tested with sensitive and specific techniques such as IFA[16–18] and WB. All the sera were negative for HIV antibodies, a result similar to that found in France, Japan and the US.[1,13] These facts indicate that AIDS virus is not transmitted through the use of immunoglobulins unless the globulins are not properly handled during production.

Nowadays, industrial manufacture of human blood products mostly apply the cryoethanol technique (Cohn's method). From the data available, the possibility of transmitting AIDS by immunoglobulins made in this way is very slight because laboratory experiments have proven that ethanol can inactivate HIV.[19–23] But this does not apply to other blood products such as cryoprecipitated and condensed VIII and IX factors. Zengyi[24] examined 18 patients with haemophilia from Zhejiang Province in China who had received injections of VIII Factor imported from the US and detected four cases positive for seral HIV antibodies. This was the first confirmed report that HIV has been imported into China. The Chinese Government has now prohibited the importation of foreign blood products and laid down regulations on the surveillance of AIDS,[25] both important control measures. Although current data cannot confirm or deny whether immunoglobulins transmit HIV, it is a good policy for people to be on the alert for this disease. According to the current situation in this country, the Chinese Government has listed six at-risk population groups, with users of foreign blood products being at greatest risk and becoming an important focus for the detection of seral HIV antibodies.[26] Immunoglobulins have been broadly used, therefore long-term follow-up surveys and epidemiological investigations should be done, especially on recipients of large doses of HIV positive immunoglobulins to confirm their safety.

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United States Patent [19]

Neurath et al.

[11] Patent Number: 4,820,805

[45] Date of Patent: Apr. 11, 1989

[54] **UNDENATURED VIRUS-FREE TRIALKYL PHOSPHATE TREATED BIOLOGICALLY ACTIVE PROTEIN DERIVATIVES**

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[73] Assignee: New York Blood Center, Inc., New York, N.Y.

[21] Appl. No.: 726,200

[22] Filed: Apr. 22, 1985

Related U.S. Application Data

[60] Continuation of Ser. No. 514,375, Jul. 14, 1983, Pat. No. 4,540,573, which is a division of Ser. No. 631,675, Jul. 17, 1984, Pat. No. 4,764,369.

[51] Int. Cl.[4] C07K 15/00; A61K 37/02

[52] U.S. Cl. 530/410; 424/89; 530/391; 530/406; 530/808; 530/829

[58] Field of Search 424/89; 530/406, 410, 530/391, 808, 829

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Attorney, Agent, or Firm—Sprung Horn Kramer & Woods

[57] ABSTRACT

A mammalian blood protein-containing composition such as whole blood, plasma, serum, plasma concentrate, cryoprecipitate, cryosupernatant, plasma fractionation precipitate or plasma fractionation supernatant substantially free of hepatitis and other lipid coated virnses with the yield of protein activity to total protein being at least 80% is disclosed. The protein-containing composition is contacted with di- or trialkylphosphate, preferably a mixture of trialkylphosphate and detergent, usually followed by removal of the di- or trialkylphosphate.

3 Claims, 2 Drawing Sheets

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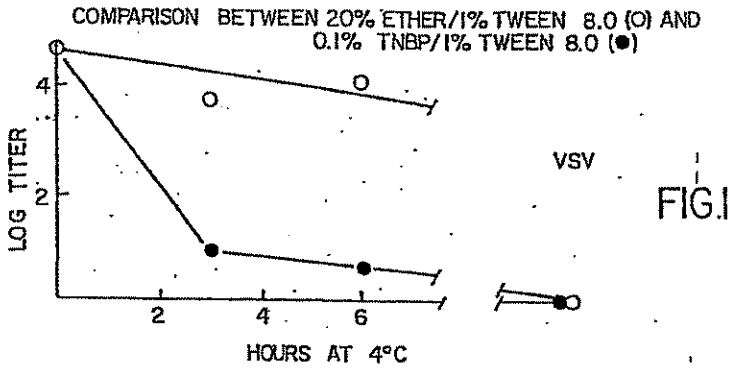
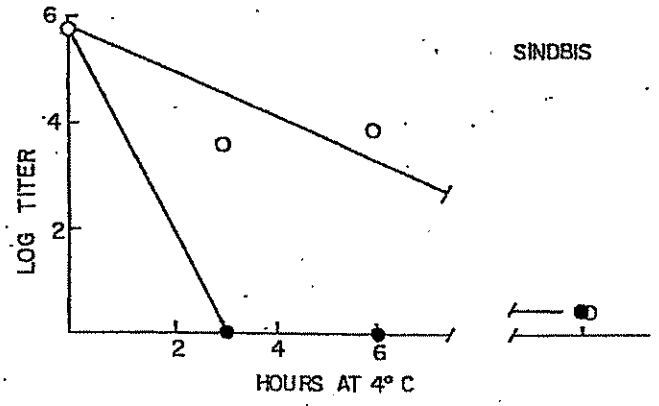


FIG.1



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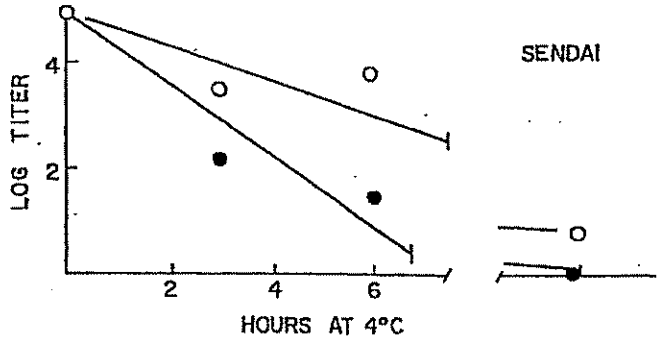

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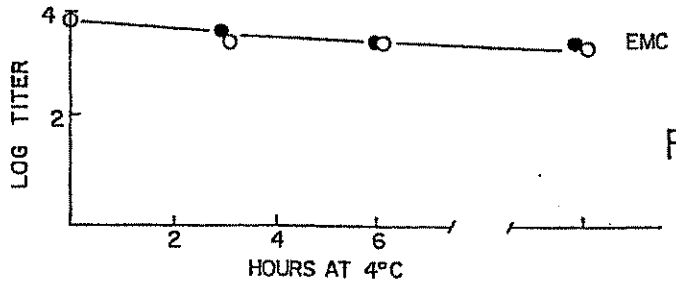

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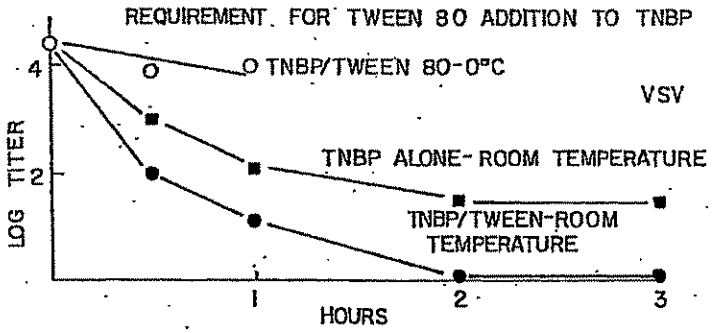

FIG.5

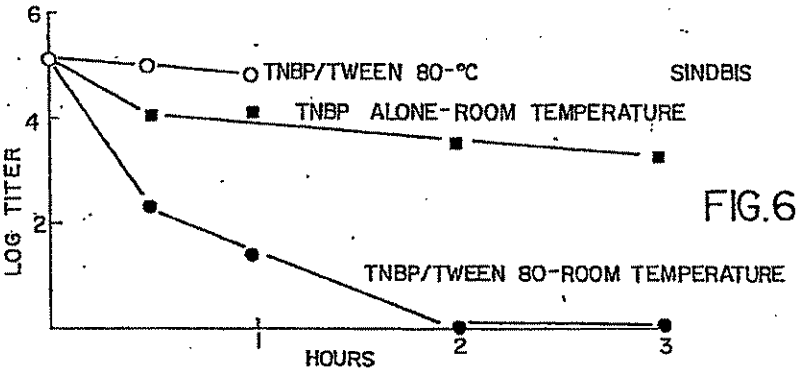

FIG.6

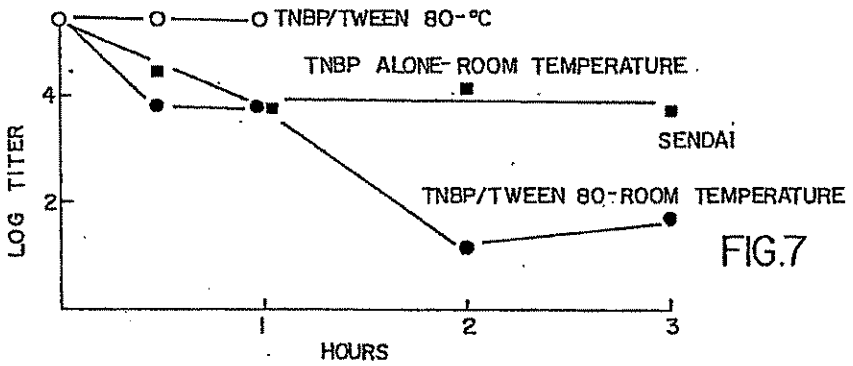

FIG.7

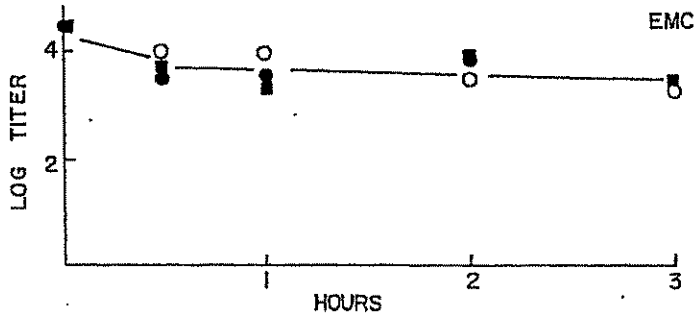

FIG.8

TNBP/TWEEN 80-°C
TNBP/TWEEN 80-ROOM TEMPERATURE
TNBP/TWEEN 80 ALONE-ROOM TEMPERATURE--.

UNDENATURED VIRUS-FREE TRIALKYL PHOSPHATE TREATED BIOLOGICALLY ACTIVE PROTEIN DERIVATIVES

This is a continuation of pending application Ser. No. 514,375, filed July 14, 1983, now U.S. Pat. No. 4,540,573. A division of Ser. No. 514,375 was filed July 17, 1984, Ser. No. 631,675, now U.S. Pat. No. 4,764,369.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to undenatured virus-free biologically active protein-containing compositions. More especially, this invention relates to the inactivation of viruses, especially lipid coated viruses, e.g., hepatitis B in human blood, blood component, blood plasma or any fraction, concentrate or derivative thereof containing blood proteins or non-blood sources including normal or cancer cells, the exudate from cancer or normal cells grown in culture, hybridomas, in products from gene splicing (DNA), etc.; by the use of di- or trialkyl phosphates, and to the resultant products. In particular, this invention relates to blood plasma or other plasma protein-containing compositions which are to be rendered substantially free of hepatitis B and/or non-A and non-B hepatitis or other viral infectivity, such blood plasma or fractions thereof having valuable labile proteins, such as, for example, factor VIII.

2. DISCUSSION OF PRIOR ART

Numerous attempts have been made to inactivate viruses such as hepatitis B virus (HBV) in mammalian, especially human, blood plasma. It is the practice in some countries to effect inactivation of the hepatitis B virus in the blood plasma by contacting the plasma with a viral inactivating agent of the type which crosslinks with the proteinaceous portion of hepatitis B virus, or which interacts with the nucleic acid of the virus. For instance, it is known to attempt to inactivate hepatitis B virus by contact with an aldehyde such as formaldehyde whereby crosslinking to the protein is effected and the hepatitis B virus is inactivated. It is also known to effect inactivation of the virus by contact with beta-propiolactone (BPL), an agent which acts on the nucleic acid of the virus. It is further known to use ultraviolet (UV) light, especially after a beta-propiolactone treatment.

Unfortunately, these agents often alter, denature or destroy valuable protein components especially so-called "labile" blood coagulation factors of the plasma under conditions required for effective inactivation of virus infectivity. For instance, in such inactivation procedures, factor VIII is inactivated or denatured to the extent of 50–90% or more of the factor VIII present in the untreated plasma. Because of the denaturing effects of these virus inactivating agents, it is necessary in the preparation of derivatives for administration to patients to concentrate large quantities of plasma so that the material to be administered to the patient once again has a sufficient concentration of the undenatured protein for effective therapeutic treatment. This concentration, however, does not affect reduction of the amount of denatured protein. As a result, the patient not only receives the undenatured protein but a quantity of denatured protein often many times that of the undenatured protein.

For instance, in the inactivation of hepatitis B virus in human blood plasma by beta-propiolactone, there is

obtained as a result thereof, a plasma whose factor VIII has been 75% inactivated. The remaining 25% of the factor VIII is therefore present in such a small concentration, as a function of the plasma itself, that it is necessary to concentrate large quantities of the factor VIII to provide sufficient concentration to be of therapeutic value. Since such separation techniques do not efficiently remove denatured factor VIII from undenatured factor VIII, the material administered to the patent may contain more denatured protein than undenatured protein. Obviously, such inactivation is valuable from a standpoint of diminishing the risk of hepatitis virus infection. However, it requires the processing of large quantities of plasma and represents significant loss of valuable protein components. Furthermore, administration of large amounts of denatured proteins may render these antigenic to the host and thus give rise to autoimmune diseases, or perhaps, rheumatoid arthritis.

The loss of these valuable protein components is not limited to factor VIII, one of the most labile of the valuable proteins in mammalian blood plasma. Similar protein denaturation is experienced in respect of the following other valuable plasma components: coagulation factors II, VII, IX, X; plasmin, fibrinogen (factor I) IgM, hemoglobin, interferon, etc.

Factor VIII, however, it denatured to a larger extent than many of the other valuable proteins present in blood plasma.

As a result of the foregoing, except in the processing of serum albumin, a stable plasma protein solution which can withstand pasteurization, it is largely the practice in the United States in respect of the processing of blood proteins to take no step in respect of the sterilization for inactivation of viruses. As a result, recipients of factor VIII, gamma-globulin, factor IX, fibrinogen, etc., must accept the risk that the valuable protein components being administered may be contaminated with hepatitis viruses as well as other infectious viruses. As a result, these recipients face the danger of becoming infected by these viruses and having to endure the damage which the virus causes to the liver and other organ systems and consequent incapacitation and illness, which may lead to death.

The BPL/UV inactivation procedure discussed above has not so far been adopted in the United States for numerous reasons, one of which lies in the fact that many researchers believe the BPL is itself deleterious since it cannot be removed completely following the inactivation and thus may remain in plasma and plasma derivatives. BPL has been shown to be carcinogenic in animals and is dangerous even to personnel handling it.

Other methods for the inactivation of hepatitis B virus in the plasma are known, but are usually impractical. One method involves the addition of antibodies to the plasma whereby an immune complex is formed. The expense of antibody formation and purification add significantly to the cost of the plasma production; furthermore, there is no assurance that a sufficient quantity of hepatitis B or non-A, non-B virus is inactivated. There is currently no test for non-A, non-B antibodies (although there is a test of the virus); hence, it is not possible to select plasma containing high titers of anti non-A, non-B antibody.

It is to be understood that the problems of inactivation of the viruses in plasma are distinct from the problems of inactivation of the viruses themselves due to the copresence of the desirable proteinaceous components of the plasma. Thus, while it is known how to inactivate

the hepatitis B virus, crosslinking agents, for example, glutaraldehyde, nucleic acid reacting chemicals, for example BPL or formaldehyde, or oxidizing agents, for example chlorox, etc., it has been believed that these methods are not suitable for the inactivation of the virus in plasma due to the observation that most of these activating agents (sodium hypochlorite, formaldehyde, beta-propiolactone) denatured the valuable proteinaceous components of the plasma.

U.S. Pat. No. 4,315,919 to Shanbrom describes a method of depyrogenating a proteinaceous biological or pharmaceutical product by contacting such proteinaceous product with non-denaturing amphiphile.

U.S. Pat. No. 4,314,997 to Shanbrom describes a method of reducing pyrogenicity, hepatitis infectivity and clotting activation of a plasma protein product by contacting the product with a non-denatured amphiphile.

Both Shanbrom '919 and '997 contemplate the use of a non-ionic detergent, for example, "Tween 80" as the amphiphile. It will be shown hereinafter that treatment with "Tween 80" by itself is relatively ineffective as a viral inactivating agent.

U.S. Pat. No. 3,962,421 describes a method for the disruption of infectious lipid-containing viruses for preparing sub-unit vaccines by contacting the virus in an aqueous medium with a wetting agent and a trialkylphosphate. Such aqueous medium is defined as allantonic fluid, tissue culture fluid, aqueous extract or suspension of central nervous system tissue, blood cell eluate and an aqueous extract or suspension of fowl embryo. The patent does not describe hepatitis, nor is it concerned with preparation of blood derivatives containing labile blood protein substantially free of viral infectivity. It is only concerned with disrupting the envelope of lipid containing viruses for the production of vaccines and not with avoiding or reducing protein denaturation en route to a blood derivative.

Problems may also exist in deriving valuable proteins from non-blood sources. These sources include, but are not limited to, mammalian mil, ascitic fluid, saliva, placental extracts, tissue culture cell lines and their extracts including transformed cells, and product of fermentation. For instance, the human lymphoblastoid cells have been isolated which produce alpha interferon. However, the cell line in commercial use today contains Epstein-Barr virus genes. It has been a major concern that the use of interferon produced by these cells would transmit viral infection or induce viral caused cancerous growth.

The present invention is directed to achieving three goals, namely, (1) a safe, (2) viral inactivated protein-containing composition, (3) without incurring substantial protein denaturation. As shown above, these three goals are not necessarily compatible since, for example, beta-propiolactone inactivates viral infectivity, but is unsafe and substances such as formaldehyde inactivate viruses, but also substantially denaturate the valuable plasma proteins, for example, factor VIII.

It, therefore, became desirable to provide a process for obtaining protein-containing compositions which does not substantially denature the valuable protein components therein and which does not entail the use of a proven carcinogenic agent. More especially, it is desirable to provide blood protein-containing compositions in which substantially all of the hepatitis viruses and other viruses present are inactivated and in which denatured protein such as factor VIII account for only a small amount of the total amount of these proteins in the blood protein-containing composition.

It is a further object to provide products from cancer or normal cells or from fermentation processes following gene insertion which are substantially free of virus, especially lipid-containing viruses.

SUMMARY OF THE INVENTION

It has now been discovered, quite surprisingly, that while most of the viral inactivating agents denature factor VIII and other valuable blood plasma proteins, that not all viral inactivating agents have such effect. It has been discovered that a protein-containing composition such as whole blood, blood cell proteins, blood plasma, a blood plasma fractionation precipitate, a blood plasma fractionation supernatant, cryoprecipitate, cryosupernatant, or portion or derivative thereof or serum or a non-blood product produced from normal or cancerous cells (e.g. via recombinant DNA technology) is contacted for a sufficient period of time with a dialkylphosphate or a trialkylphosphate that lipid containing viruses such as the hepatitis viruses present in the composition are virtually entirely inactivated without substantial denaturation of proteins therein. By contacting blood protein mixture or concentrate thereof or fraction thereof with a di- or trialkylphosphate, followed by removal of the di- or trialkylphosphate, hepatitis viruses can be substantially inactivated, e.g., to an inactivation of greater than 4 logs, while realizing a yield of protein activity to total protein of at least 80%.

By such procedures there is provided a blood protein-containing composition such as mammalian whole blood, blood cell derivatives (e.g., hemoglobin, alpha-interferon, T-cell growth factor, platelet-derived growth factor, etc.), plasminogen activator, blood plasma, blood plasma fraction, blood plasma precipitate (e.g., cryoprecipitate, ethanol precipitate or polyethylene glycol precipitate), or supernatant (e.g., cryosupernatant, ethanol supernatant or polyethylene glycol supernatant), characterized by the presence of one or more blood proteins such as labile blood factor VIII having a total yield of protein activity to total protein of at least 80%, preferably at least 85%, more preferably 95% and most preferably 98% to 100%, said blood protein-containing composition having greatly reduced or virtually no hepatitis viruses. Virus in a serum is determined by infectivity titrations.

By the inactivation procedure of the invention, most if not virtually all of the hepatitis viruses contained therein would be inactivated. The method for determining infectivity levels by in vivo chimpanzees is discussed by Prince, A. M., Stephen, W., Brotman, B. and van den Ende, M. C., "Evaluation of the Effect of Beat-propiolactone/Ultraviolet Irradiation (BPL/UV) Treatment of Source Plasma on Hepatitis Transmission by factor IV Complex in Chimpanzees, Thrombosis and Haemostasis", 44: 138–142, 1980.

The hepatitis virus is inactivated by treatment with the di- or trialkylphosphate described herein, and is not inactivated because of inclusion in the plasma of antibodies which bind with the hepatitis viruses and form immune complexes.

Inactivation of virus is obtained to the extent of at least "4 logs", i.e., virus in a serum is totally inactivated to the extent determined by infectivity studies where that virus is present in the untreated serum in such a concentration that even after dilution to 10^4, viral activity can be measured.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows virus inactivation as a function of log titer value versus time for VSV virus (vesicular stomatitis virus) treated according to the present invention and treated with ether/Tween 80. The lower log titer for treatment according to the present invention indicates greater virus inactivation;

FIG. 2 shows virus inactivation as a function of log titer value versus time for Sindbis virus treated according to the present invention and treated with ether/-Tween 80;

FIG. 3 shows virus inactivation as a function of log titer value versus time for Sendai virus treated according to the present invention and treated with ether/-Tween 80;

FIG. 4 shows virus inactivation as a function of log titer value versus time for EMC virus (a non-lipid coated virus) treated according to the present invention and treated with ether/Tween 80;

FIG. 5 is a plot of log titer value versus hours for VSV virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature);

FIG. 6 is a plot of log titer value versus hours for Sindbis virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature);

FIG. 7 is a plot of log titer value versus hours for Sendai virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature); and

FIG. 8 is a plot of log titer value versus hours for EMC virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature).

The Sindbis, Sendai and VSV viruses are typical lipid containing viruses and are used herein to determine the effect of di- or trialkylphosphate on lipid coated viruses generally.

DETAILED DESCRIPTION OF THE INVENTION

Blood is made up of solids (cells, i.e., erythrocytes, leucocytes, and thrombocytes) and liquid (plasma). The cells contain potentially valuable substances such as hemoglobin, and they can be induced to make other potentially valuable substances such as interferons, growth factors, and other biological response modifiers. The plasma is composed mainly of water, salts, lipids and proteins. The proteins are divided into groups called fibrinogens, serum globulins and serum albumins. Typical antibodies (immune globulins) found in human blood plasma includes those directed against infectious hepatitis, influenza H, etc.

Blood transfusions are used to treat anemia resulting from disease or hemorrhage, shock resulting from loss of plasma proteins or loss of circulating volume, diseases where an adequate level of plasma protein is not maintained, for example, hemophilia, and to bestow passive immunization.

Whole blood must be carefully typed and cross matched prior to administration. Plasma, however, does not require prior testing. For certain applications, only a proper fraction of the plasma is required, such as factor VIII for treatment of hemophilia or von Willebrand's disease.

With certain diseases one or several of the components of blood may be lacking. Thus, the administration of the proper fraction will suffice, and the other components will not be "wasted" on the patient; the other fractions can be used for another patient. The separation of blood into components and their subsequent fractionation allows the proteins to be concentrated, thus permitting concentrates to be treated. Of great importance, too, is the fact that the plasma fractions can be stored for much longer periods than whole blood and they can be distributed in the liquid, the frozen, or the dried state. Finally, it allows salvaging from blood banks the plasma portions of outdated whole blood that are unsafe for administration as whole blood.

Proteins found in human plasma include prealbumin, retinol-binding protein, albumin, alpha-globulins, beta-globulins, gamma-globulins (immune serum globulins), the coagulation proteins (antithrombin III, prothrombin, plasminogen, antihemophilic factor-factor VIII, fibrin-stabilizing factor-factor XIII, fibrinogen), immunoglobins(immunoglobulins C, A, M, D, and E), and the complement components. There are currently more than 100 plasma proteins that have been described. A comprehensive listing can be found in "The Plasma Proteins", ed. Putnam, F. W., Academic Press, New York (1975).

Proteins found in the blood cell fraction include hemoglobin, fibronectin, fibrinogen, enzymes of carbohydrate and protein metabolism, etc. In addition, the synthesis of other proteins can be induced, such as interferons and growth factors.

A comprehensive list of inducible leukocyte proteins can be found in Stanley Cohen, Edgar Pick, J. J. Oppenheim, "Biology of the Lymphokines", Academic Press, New York (1979).

Blood plasma fractionation generally involves the use of organic solvents such as ethanol, ether and polyethylene glycol at low temperatures and at controlled pH values to effect precipitation of a particular fraction containing one or more plasma proteins. The resultant supernatant can itself then be precipitated and so on until the desired degree of fractionation is attained. More recently, separations are based on chromatographic processes. An excellent survey of blood fractionation appears in *Kirk-Othmer's Encyclopedia of Chemical Technology*, Third Edition, Interscience Publishers, Volume 4, pages 25 to 62, the entire contents of which are incorporated by reference herein.

The major components of a cold ethanol fractionation are as follows:

Fraction	Proteins
I	fibrinogen; cold insoluble globulin; factor VIII; properdin
II and III	IgG; IgM; IgA; fibrinogen; beta-lipoprotein; prothrombin; plasminogen; plasmin inhibitor; factor V; factor VII; factor IX; factor X; thrombin; antithrombin; isoagglutinins; ceruloplasmin; complement C'1, C'3
IV-1	alpha$_1$-lipoprotein, cerulotplasmin; plasmin-inhibitor; factor IX; peptidase; alpha-and-beta-globulins
IV-4	transferrin; thyroxine binding globulin; serum esterase; alpha$_1$-lipoprotein; albumin; alkaline phosphatase
V	albumin; alpha-globulin
VI	alpha$_1$-acid glycoprotein; albumin

The above fractionation scheme can serves as a basis for further fractionations. Fraction II and III, for example, can be further fractionated to obtain immune serum globulin (ISG).

Another fractionation scheme involves use of frozen plasma which is thawed into a cryoprecipitate containing AHD (antihemophilic factor) and fibronectin and a cryosupernatant. The cryoprecipitate is then fractionated into fibronectin and AHF.

Polyethylene glycol has been used to prepare high purity AHD and non-aggregated ISG.

High risk products with respect to the transmission of hepatitis B and non-A, non-B are fibrinogen, AHF and prothrombin complex, and all other blood protein preparations except immune serum globulin and, because they are pasteurized, albumin solutions. Hepatitis tests presently available can indicate the presence of hepatitis B surface antigen, but there is presently no screening test for non-A, non-B hepatitis.

The present invention is directed to contacting with di- or trialkylphosphate a blood protein-containing composition such as whole mammalian blood, blood cells thereof, blood cell proteins, blood plasma thereof, precipitate from any fractionation of such plasma, supernatant from any fractionation of such plasma, cryoprecipitate, cryosupernatant or any portions or derivatives of the above that contain blood proteins such as, for example, prothrombin complex (factors II, VII, IX and X) and cryoprecipitate (factors I and VIII). The present invention is also concerned with contacting di- or trialkylphosphate with a serum containing one or more blood proteins. Furthermore, the present invention is directed to contacting di- or trialkylphosphate with a blood protein-containing fraction containing at least one blood protein such as the following: factor II, factor VII, factor VIII, factor IX, factor X, fibrinogen and IgM. additionally, the present invention concerns contacting a cell lysate or proteins induced in blood cells with di- or trialkylphosphate.

Such blood protein-containing composition is contacted with a dialkylphosphate or a trialkylphosphate having alkyl groups which contain 1 to 10 carbon atoms, especially 2 to 10 carbon atoms. Illustrative members of trialkylphosphates for use in the present invention include tri-(n-butyl)phosphate, tri-(t-butyl)-phosphate, tri-(n-hexyl)phosphate, tri-(2-ethylhexyl)-phosphate, tri-(n-decyl)phosphate, just to name a few. An especially preferred trialkylphosphate is tri-(n-butyl)phosphate. Mixtures of different trialkylphosphates can also be employed as well as phosphates having alkyl groups of different alkyl chains, for example, ethyl, di(n-butyl) phosphate. Similarly, the respective dialkylphosphates can be employed including those of different alkyl group mixtures of dialkylphosphate. Furthermore, mixtures of di- and trialkylphosphates can be employed.

Di- or Trialkylphosphates for use in the present invention are employed in an amount between about 0.01 mg/ml and about 100 mg/ml, and preferably between about 0.1 mg/ml and about 10 mg/ml.

The di- or trialkylphosphate can be used with or without the addition of wetting agents. It is preferred, however, to use di- or trialkylphosphate in conjunction with a wetting agent. Such wetting agent can be added either before, simultaneously with or after the di- or trialkylphosphate contacts the blood protein-containing composition. The function of the wetting agent is to enhance the contact of the virus in the blood protein-containing composition with the di- or trialkylphosphate. The wetting agent alone does not adequately inactivate the virus.

Preferred wetting agents are non-toxic detergents. Contemplated nonionic detergents include those which disperse at the prevailing temperature at least 0.1% by weight of the fat in an aqueous solution containing the same when 1 gram detergent per 100 ml of solution is introduced therein. In particular there is contemplated detergents which include polyoxyethylene derivatives of fatty acids, partial esters of sorbitol anhydrides, for example, those products known commercially as "Tween 80", "Tween 20" and "polysorbate 80" and nonionic oil soluble water detergents such as that sold commercially under the trademark "Triton X 100" (oxyethylated alkylphenol). Also contemplated is sodium deoxychloate as well as the "Zwittergents" which are synthetic zwitterionic detergents known as "sulfobetaines" such as N-dodecyl-N, N-dimethyl-2-ammonio-1ethane sulphonate and its congeners or nonionic detergents such as octyl-beta-D-glucopyranoside.

Substances which might enhance the effectiveness of alkylphosphates include reducing agents such as mercaptoethanol, dithiothreitol, dithioerythritol, and dithiooctanoic acid. Suitable nonionic surfactants are oxyethylated alkyl phenols, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene acids, polyoxyethylene alcohols, polyoxyethylene oils and polyoxyethylene oxypropylene fatty acids. Some specific examples are the following:

alkylphenoxypolyethoxy (30) ethanol
polyoxyethylene(2) sorbitan monolaurate
polyoxyethylene (20) sorbitan monopalmitate
polyoxyethylene (20) sorbitan monostearate
polyoxyethylene (20) sorbitan tristearate
polyoxyethylene (20) sorbitan monooleate
polyoxyethylene (20) sorbitan trioleate
polyoxyethylene (20) palmitate
polyoxyethylene (20) lauryl ether
polyoxyethylene (20)cetyl ether
polyoxyethylene (20) stearyl ether
polyoxyethylene (20) oleyl ether
polyoxyethylene (25) hydrogenated castor oil
polyoxyethylene (25) oxypropylene monostearate

The amount of wetting agent, if employed, is not crucial, for example, from about 0.001% to about 10%, preferably about 0.01 to 1.5%, can be used.

Di- and trialkylphosphates may be used in conjunction with other inactivating agents such as alcohol or ethers with or without the copresence of wetting agents in accordance with copending application Ser. No. 368,250 entitled "Sterilized Plasma and Plasma Derivatives and Process Therefor", assigned to the assignee hereof.

The ether or alcohol can be added in an amount of 1 to 50%, preferably 5 to 25% by weight, based on the volume of blood plasma, or concentrate or other blood plasma protein-containing composition to be treated.

Particularly contemplated ethers for inactivation use in accordance with the invention are those having the formula

$$R^1—O—R^2$$

wherein

R^1 and R^2 are independently C_1–C_{18} alkyl or alkenyl which can contain an O or S atom in the chain, preferably C_1–C_8 alkyl or alkenyl. Especially contemplated ethers are dimethyl ether, diethyl ether, ethyl propyl ether, methyl-butyl ether, methyl isopropyl ether and methyl isobutyl ether.

Alcohols contemplated include those of the formula

R^3OH

wherein

R^3 is a C_1 to C_{18} alkyl or alkenyl radical which can contain one or more oxygen or sulfur atoms in the chain and which can be substituted by one or more hydroxyl groups.

Especially contemplated alcohols are those where the alkyl or alkenyl group is between 1 and 8 atoms. Particularly contemplated alcohols include methanol, ethanol, propanol, isopropanol, n-butanol, isobutanol, n-pentanol and the isopentanols. Also contemplated are compounds such as ethylene glycol, 1,2-propylene glycol 1,3-propane diol, 1,4-butanediol, 2-hydroxy isobutanol (2-methy, 1,2-dihydroxypropane).

Treatment of blood protein-containing compositions with trialkylphosphate is effected at a temperature between −5° C. and 70°, preferably between 0° C. and 60° C. The time of such treatment (contact) is for at least 1 minute, preferably at least 1 hour and generally 4 to 24 hours. The treatment is normally effective at atmospheric pressure, although subatmospheric and superatmospheric pressures can also be employed.

Normally, after the treatment, the trialkylphosphate and other inactivating agents, for example, ether, are removed, although such is not necessary in all instances, depending upon the nature of the virus inactivating agents and the intended further processing of the blood plasma protein-containing composition.

To remove ether from plasma the plasma is generally subjected to a temperature of 4° C. to 37° C. with a slight vacuum imposed to draw off residual ether. Preferably means are provided to spread the plasma as a thin film to insure maximum contact and removal of the ether. Other methods for removal of ether in activating agents include:

(1) bubbling of nitrogen gas;

(2) diafiltration using ether insoluble, e.g. "TEFLON", microporous membranes which retain the plasma proteins;

(3) absorption of desired plasma components on chromatographic or affinity chromographic supports;

(4) precipitation, for example, by salting out of plasma proteins;

(5) lyophilization, etc.

When alcohol or nonionic detergents are employed with the trialkylphosphate they are removed by (2) to (5) above.

Di- or trialkylphosphate can be removed as follows:

(a) Removal from AHF can be effected by precipitation of AHF with 2.2 molal glycine and 2.0M sodium chloride

(b) Removal from fibronectin can be effected by binding the fibronectin on a column of insolubilized gelatin and washing the bound fibronectin free of reagent.

Generally speaking, any ether present is initially removed prior to removal of any detergent. The ether may be recovered for reuse by the use of suitable distillation/condenser systems well known to the art.

Alcohol is normally removed together with detergent. If the detergent includes both alcohol and ether, the ether is normally removed before the alcohol.

The process of the invention can be combined with still other modes of inactivating viruses including those for non-lipid coated viruses. For instance, a heating step can be effected in the presence of a protein stabilizer, e.g., an agent which stabilizes the labile protein (AHF) against inactivation by heat. Moreover, the heating can be carried out using stabilizers which also tend to protect all protein, including components of the virus, against heat if the heating is carried out for a sufficient length of time, e.g., at least 5 hours and preferably at least 10 hours at a temperature of 50°–70° C., especially 60° C. By such mode the virus is preferentially inactivated, nevertheless, while the protein retains a substantial amount, e.g., ≧80% of its protein activity. Of course, the best treatment can also be carried out simultaneously with the alkyl phosphate treatment.

The treatment of plasma or its concentrates, fractions or derivatives in accordance with the present invention can be effected using di- or trialkylphosphate immobilized on a solid substance. The same can be fixed to a macro-molecular structure such as one of the type used as a backbone for ion exchange reactions, thereby permitting easy removal of the trialkylphosphate from the plasma or plasma concentrate. Alternatively the phosphate can be insolubilized and immobilized on a solid support such as glass beads, etc., using silane or siloxane coupling agents.

The method of the present invention permits the pooling of human blood plasma and the treatment of the pooled human blood plasma in the form of such pooled plasma. It also permits the realization of blood product derivatives such as factor VIII, gamma globulin, factor IX or the prothrombin complex (factors II, VII, IX, X), fibrinogen and any other blood derivative including HBsAg used for the preparation of HBV vaccine, all of which contain little or no residual infective hepatitis or other viruses.

The present invention is directed, inter alia, to producing a blood plasma blood plasma fractions, etc., which is substantially free of infectious virus, yet which contains a substantial amount of viable (undenatured) protein. More particularly, the present invention is directed to inactivation of lipid-containing virus and preferentially inactivation of hepatitis B and non-B, non-A virus. Other viruses inactivated by the present invention include, for example, cytomegaloviruses, Epstein Barr viruses, lactic dehydrogenase viruses, herpes group viruses, rhabdoviruses, leukoviruses, myxoviruses, alphaviruses, Arboviruses (group B), paramyxoviruses, arenaviruses, and coronaviruses.

According to the present invention, there is contemplated a protein-containing composition—a product produced from normal or cancerous cells or by normal or cancerous cells (e.g., via recombinant DNA technology), such as mammalian blood, blood plasma, blood plasma fractions, precipitates from blood fractionation and supernatants from blood fractionation having an extent of inactivation of virus greater than 4 logs of virus such as hepatitis B and non-A, non-B, and having a yield of protein activity to total protein of at least 80%, preferably at least 95% and most preferably 98% to 100%.

Further contemplated by the present invention is a composition containing factor VIII which is substantially free of hepatitis virus to the extent of having an inactivation of greater than 4 logs of the virus and a yield of protein activity to total protein of at least 80%, preferably at least 85%, more preferably at least 95% and most preferably 98% to 100%.

The process of the present invention has been described in terms of treatment of plasma, plasma frac-

tions, plasma concentrates or components thereof. The process, however, is also useful in treating the solid components of blood, lysates or proteins secreted by cells. Thus, also contemplated are treatment of platelet concentrates, white cell (leukocyte) concentrates, and leukocyte-poor packed red cells as well as platelet rich plasma, platelet concentrates and platelet poor plasma including packed cell masses comprising the white buffy coat consisting of white blood cells above packed red cells. Also contemplated is the treatment of masses containing concentrates of granulocytes, monocytes, interferon, and transfer factor.

One can treat plasma itself according to the present invention or fresh frozen plasma, thawed frozen plasma, cryoprecipitate, cryosupernatants or concentrates from frozen plasma as well as dilution products thereof.

By the same manipulation steps discussed above virus present in products of normal or cancerous cells can be inactivated while retaining labile protein activity in such products. For instance, by the same di- or trialkylphosphate treatment one can inactivate products produced using normal or cancer cells, the exudate from normal or cancerous cells, hybridomas, and products produced by gene splicing. Such treatment does not substantially adversely affect the desired protein. Cells used for production of desired protein can, of course, be mammalian as well as non-mammalian cells.

Factor VIII and factor IX consultant activities are assayed by determining the degree of correction in APTT time of factor VIII—and factor IX—deficient plasma, respectively. J. G. Lenahan, Phillips and Phillips, *Clin. Chem.*, Vol. 12, page 269 (1966).

The activity of proteins which are enzymes is determined by measuring their enzymatic activity. Factor IX's activity can be measured by that technique.

Binding proteins can have their activities measured by determining their kinetics and affinity of binding to their natural substrates.

Lymphokine activity is measured biologically in cell systems, typically by assaying their biological activity in cell cultures.

Protein activity generally is determined by the known and standard modes for determining the activity of the protein or type of protein involved.

In order to more fully illustrate the nature of the invention and the manner of practicing the same, the following non-limiting examples are presented.

EXAMPLE 1

AHF solutions were incubated with 0.1% TNPB plus 1% Tween 80 for 18 hours at 4° C. These solutions were initially contacted with VSV virus, Sindbis virus and Sendai virus and thereafter brought in contact with an aqueous solution containing 0.1 weight percent of tri(n-butyl) phosphate (TNBP) and 1.0 weight percent detergent (Tween 80), with the following resultant virus inactivations: 4.7 logs of vesicular stomatitis virus (VSV), 5.8 logs of Sindbis virus, and 5.0 logs of Sendai virus. The virus was added just prior to the addition of the TNBP-Tween 80. The yield of AHD (Labile protein/total protein) was found to be 86%.

Controls in which TNBP and Tween 80 were omitted showed little if any viral inactivation.

The results for Example 1 are shown below in Table I:

TABLE I

Temperature	Time (Hrs)	AHF U/mL	AHF % Yld	Log Titer Virus VSV	Sindbis	Sendai
4° C.	Start	10.4	(100)	4.7	5.8	5.0
	3	—	—	0.9	−0.4	2.2
	6	—	—	0.6	−0.5	1.5
	18	8.9	86	<−0.5	0.5	−0.5

In FIG. 1, FIG. 2, and FIG. 3, the results of Example 1 are plotted and compared to virus inactivation with ether (20%)/Tween 80 (1%). It is seen that for VSV (FIG. 1). Sindbis (FIG. 2) and Sendai (FIG. 3), inactivation was greater (lower log titer value) for treatment according to the present invention (with TNBP) than with ether/Tween 80 treatment.

In Table II, the effect of a "Tween 80" alone in the inactivation of viruses is shown. The data shows that little if any inactivation is due to "Tween 80".

TABLE II

EFFECT OF TWEEN 80 (1%) ALONE ON VIRUS INACTIVATION

Experiment	Temperature (°C.)	Duration (Hrs)	Inactivation (log#) VSV	Sindbis	Sendai	EMC
1	0° C.	3	0.3	0.0	0.0	0.4
2	0° C.	18	ND*	−0.1	0.7	0.5
	22° C.	18	ND*	−0.1	−0.3	0.0

#log titer control minus log titer treated
*not done

Example 2

Example 1 was repeated, but at 22° C. The results for Example 2 are summarized below in Table III:

TABLE III

Temperature	Time (Hrs)	AHF U/mL	AHF % Yld	Log Titer Virus VSV	Sindbis	Sendai
22° C.	Untreated	8.3	(100)	4.4	5.1	5.0
	3	8.2	99	<−0.4	<−0.5	1.8

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be had to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

We claim:

1. A process for obtaining a protein-containing composition which is substantially free of lipid-containing viruses without incurring substantial denaturation of any labile proteins contained therein, comprising contacting said protein-containing composition with an effective amount of di- or trialkylphosphate for a sufficient period of time, wherein said protein-containing composition is the product of a cancerous cell or the product of gene splicing.

2. A process according to claim 1, wherein the protein-containing composition is a product of a cancerous cell.

3. A process according to claim 1, wherein the protein-containing composition is a product of gene splicing.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,820,805
DATED : Apr. 11, 1989
INVENTOR(S) : Neurath et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Sheets 1 and 2 of Drawings, Figures 1, 2, 3, 4, 5 and 6	Darken in the circle on the y-axis
Sheet 2 of Drawings, Figures 6, 7 and 8	Delete "TNBP/TWEEN 80-^{o}C" and substitute --TNBP/TWEEN 80-0^{o}C--
Col. 2, line 26	Delete "it" and substitute --is--
Col. 3, line 41	Correct spelling of --milk--
Col. 4, line 53	Correct spelling of --Beta--
Col. 8, line 17	Leave a space before --ethane--
Col. 10, line 36	After "plasma" in first instance insert --protein-containing composition such as blood,--

Signed and Sealed this

Twenty-eighth Day of August, 1990

Attest:

Attesting Officer

HARRY F. MANBECK, JR.

Commissioner of Patents and Trademarks

United States Patent [19]

Tenold

[11] Patent Number: 4,499,073

[45] Date of Patent: * Feb. 12, 1985

[54] INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

[75] Inventor: Robert A. Tenold, Benicia, Calif.

[73] Assignee: Cutter Laboratories, Inc., Berkeley, Calif.

[*] Notice: The portion of the term of this patent subsequent to Aug. 2, 2000 has been disclaimed.

[21] Appl. No.: 485,683

[22] Filed: Apr. 18, 1983

Related U.S. Application Data

[63] Continuation of Ser. No. 295,916, Aug. 24, 1981, Pat. No. 4,396,608.

[51] Int. Cl.³ A23J 1/06; A61K 39/35; A61K 37/04; C07G 7/00

[52] U.S. Cl. ..... 424/85; 424/101; 514/21; 260/112 B; 260/112 R

[58] Field of Search ..... 260/112 B, 112 R; 424/177, 85

[56] References Cited

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4,168,303	9/1979	Nishida et al.	424/85

Primary Examiner—Lionel M. Shapiro
Assistant Examiner—Robin Teskin
Attorney, Agent, or Firm—David J. Aston; Lester E. Johnson; Theodore J. Leitereg

[57] ABSTRACT

A composition is disclosed which comprises a solution in a pharmaceutically acceptable carrier of an immune serum globulin, said solution having an ionic strength and a pH to maintain the monomer content and the actual and latent anticomplement activity of the immune serum globulin such that the composition is intravenously injectable. Novel methods are disclosed for preparing the above composition.

7 Claims, No Drawings

INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

BACKGROUND OF THE INVENTION

This application is a continuation of application Ser. No. 295,916, filed Aug. 24, 1981 now U.S. Pat. No. 4,396,608.

FIELD OF THE INVENTION

This invention relates to pharmaceutical compositions comprising novel intravenously injectable immune serum globulin, to a process for its production and to its use to administer immune serum globulin intravenously for human therapy.

Intramuscularly injectable gamma globulin preparations are known. One such product is "HYPER-TET" (Cutter Laboratories, Inc., Berkeley, Calif.).

The usual intramuscular gamma globulin preparations cannot safely be administered intravenously because such administration causes an unacceptably high incidence of reactions, especially in agammaglobulinemic recipients. These reactions have been associated with a decrease in serum complement levels, apparently caused by complement binding by the administered gamma globulin. S. Barandun et al., *Vox Sang.* 7, 157–174 (1962). The ability of gamma globulin to bind complement, termed anticomplementary, is greatly increased as a result of denaturation brought about during the fractionation procedure, in particular by aggregation to high molecular weight species. The complement binding mechanism of these aggregates appears to be identical to that of antigen-antibody complexes. D. M. Marcus, *J. Immunol.* 84, 273–284 (1960). When the aggregates are removed by ultracentrifugation at 100,000×gravity, a product low in anticomplement activity is obtained which is well tolerated upon intravenous injection. Barandun et al., supra.

Several approaches have been taken to the problem of rendering gamma globulin safe for intravenous administration. All of these are dependent on eliminating its anticomplement activity. Ultracentrifugation (cited above) is technically unfeasible, and the product so derived regains its anticomplement activity upon storage. Treatment of gamma globulin with the enzyme pepsin at pH 4.0 results in proteolytic cleavage of the molecule to give a fragment of about 10,000 molecular weight which has a sedimentation coefficient in the ultracentrifuge of about 5S, A. Nisonoff et al., *Science*, 132, 1770–1771 (1960). Even though this surviving fragment retains bivalent antibody activity and lacks anticomplement activity and is well tolerated and efficacious in intravenous administration, W. Baumgarten, *Vox Sang.* 13, 84 (1967), the therapeutic effect provided is of unacceptably short duration since it is rapidly excreted, having a circulating half-life of only 18 hours, perhaps somewhat longer in agammaglobulinemic patients, compared to 19.8 days for unmodified gamma globulin. E. Merler et at., *Vox Sang.* 13, 102 (1967); B. Jager, *Arch. Intern. Med.* 119, 60 (1967). Although the much reduced half-life of pepsin treated gamma globulin is probably due in part to the drastic reduction in size of the molecule, there are indications that the rate of catabolism of gamma globulin is related to specific properties of the portion of the molecule digested by pepsin. J. L. Fahey el at., *J. Exper. Med.*, 118, 1845–1868 (1963). This portion of the molecule remains intact in the present invention. An additional disadvantage of the pepsin treatment procedure is that the pepsin which remains present is of animal origin and can stimulate antibody production, particularly upon repeated administration. C. Blatrix et al., *Presse Med.* 77, 635–637 (1969). The use of plasmin of human origin avoids this difficulty and is the basis of a different process for preparation of intravenous gamma globulin.

Treatment of gamma globulin with human plasmin results in cleavage into three components of about 50,000 molecular weight. J. T. Sgouris, *Vox Sang.* 13, 71 (1967). When sufficiently low levels of plasmin are used, only about 15 percent of the molecules are cleaved, with 85 percent remaining as intact gamma globulin. Sgouris, supra. The intact gamma globulin remaining undigested shows little anticomplement activity and has been administered intravenously without adverse reactions. J. Hinman et at., *Vox Sang.* 13, 85 (1967). The material thus prepared appears to retain in vitro and in vivo protective activity. F. K. Fitzpatrick, *Vox Sang.* 13, 85 (1967). One disadvantage of this approach is that the plasmin cannot be completely removed. Thus, degradation continues even when the material is stored at 4° C.

Incubation of gamma globulin at pH 4.0 at 37° C. for various lengths of time has been observed to reduce the anticomplement activity to low levels. It has been suggested that this result may arise from a small quantity of serum enzyme present as an impurity in the gamma globulin. Blatrix et al., supra. As with the plasmin treated gamma globulin, this "pH 4.0 gamma globulin" has been found to regain anticomplement activity, upon storage, at an unpredictable rate, so that it is necessary to assay anticomplement activity before administration to a patient. J. Malgras et al., *Rev. Franc. Trans.*, 13, 173 (1970).

Both plasmin treated gamma globulin, Hinman et al., supra, and pH 4.0 gamma globulin, H. Koblet et al., *Vox Sang.* 13, 93 (1967); J. V. Wells et al., *Austr. Ann. Med.* 18, 271 (1969); Barandun et al., *Monogr. Allergy*, Vol. 9, 39–60 (1975), Barandun et al., *Vox Sang.*, Vol. 7, 157–174 (1962), have shorter half-lives in vivo than unmodified gamma globulin. For example, the half-life in normal patients of pH 4.0 gamma globulin is about 14 days, Koblet et al., supra, while the plasmin treated material shows a half-life of 16 days, Merler et al., supra.

The Centre National de Transfusion Sanguine (C.N.T.S.) in Paris has, by careful fractionation and filtration of gamma globulin from selected fresh plasma, produced an intravenously injectable gamma globulin with low anticomplement activity. Blatrix et al., supra; ibid., *Presse Med.*, 77, 159–161 (1969); M. Steinbuch et al., *Vox Sang.* 13, 103 (1967). It is apparently not totally devoid of anticomplement activity, as it must be administered carefully and reactions do occur in some patients. Cortisone may be given prior to injection to eliminate these reactions, but the apparent incomplete removal of anticomplement activity would seem to be detrimental to its widespread use.

The effects on anticomplement activity of reduction of disulfide linkages of gamma globulin followed by reaction with a blocking agent has been investigated in the prior art. S. Barandun et al., supra, found that treatment of a 7 percent solution of gamma globulin with 0.2 M cysteamine, followed by 0.2 M iodoacetamide, resulted in almost complete loss of anticomplement activity whereas treatment with cysteamine or iodoacetamide alone did not significantly decrease anticomple-

ment activity. Because of the toxicity of iodoacetamide, these investigators did not pursue this approach to an intravenously injectable gamma globulin.

A modified immune serum globulin was described in U.S. Pat. No. 3,903,262. The immune serum globulin was rendered intravenously injectable by first reducing to —SH groups a portion of the disulfide linkages of the molecule and then alkylating the —SH groups. After the product was separated from the reaction mixture, it was sterilized. The so-produced material was intravenously injectable, substantially free from both actual and latent anticomplement activity, having substantially the biological half-life and spectrum of antibody activity of corresponding unmodified immune serum globulin.

Currently, there are several intravenously injectable gamma globulin products available outside the United States. One such product is INTRAGLOBIN of Biotest in Frankfurt. This product is made by beta-propriolactone treatment of gamma globulin (Stephan, *Vox. Sang.*, 1975, Vol. 28, pp. 422–437). The material has a molar concentration of sodium ion of about 0.18 and of chloride of about 0.27. The beta-propriolactone used in its preparation is suspected as a carcinogen.

Another intravenously injectable product is manufactured by Green Cross Corporation of Japan (U.S. Pat. No. 4,168,303). It is a lyophilized, natural gamma globulin preparation having an anticomplementary activity of less than or equal to 20 C'H50 units and 0.06–0.26 parts by weight of a neutral mineral salt such as sodium chloride.

The Swiss Red Cross has an immunoglobulin SRC for intravenous administration. SRC contains more than 80% of monomeric IgG and minor fractions of dimeric, polymeric, and fragmented IgG as well as traces of IgA and IgM. The distribution of IgG subclasses equals that of normal serum. The product is manufactured in lyophilized form and contains 3 g of protein, 5 g of saccharose and a small quantity of sodium chloride per unit. A diluent (100 ml) contains 0.9% sodium chloride.

VENOGLOBULIN (Green Cross Corporation of Japan) is prepared by treating gamma globulin with plasmin. It also contains 0.5 parts of a protein stabilizer (e.g. amino acetate) per 1 part by weight of plasmin treated gamma globulin. The product is distributed as a white powder and is dissolved in a diluent for use. The resulting solution is clear or slightly turbid and has a pH of 6.4–7.4.

An intravenously injectable gamma globulin has been developed by Schwab of Germany and contains 50 mg per ml immunoglobulin, 7 mg/ml glycine, and 7 mg/ml sodium chloride.

Schura of Germany manufactures an intravenously injectable gamma globulin by adsorption onto hydroxyethyl starch. The product is distributed as a solution having a pH of 6.7 and a conductivity of 450 mosm. and containing 2.5% glucose, 165 meq/l of sodium ion and 120 meq/l of chloride ion.

VEINOGLOBULINE is available from Institute Merieux of France. It is a plasmin-treated gamma globulin distributed as a lyophilized powder containing 5 g. of protein and enough glycine and sodium chloride to insure pH and stability. The diluent is 100 ml of water for injection containing 0.9 g. of sodium chloride or isotonic glucose.

U.S. Pat. No. 4,160,763, assigned to Behringwerke AG of Germany, is directed to an immunoglobulin for intravenous administration having reduced complement fixation made by treating an immuno globulin fraction with a low concentration of a sulfitolytic agent and/or phosphate which is sparingly soluble in water. The pH of the material is 7.0, and the product contains 0.85% sodium chloride and 2.5% (g/v) glycine prior to lyophilization.

Teijin Institute of Tokyo is the assignee of record of U.S. Pat. No. 4,059,571 for a novel immunoglobulin derivative. A water soluble composition for intravenous injection which contains the novel derivative is described. The derivative is the S-sulfonated product of cleaved interchain disulfide bonds of gamma globulin.

GLOVENIN, a pepsin-treated human immunoglobulin, is manufactured by Nihon Seigaku of Japan. Typically, a solution of the above product contains 50 mg/ml of pepsin-treated immunoglobulin, 2.25% (w/v) of aminoacetic acid, and 0.85% (w/v) sodium chloride.

Yamanouch Seiyaku is the distributor of GLOBULIN V, a dried pepsin-treated human immunoglobulin (500 mg) containing 225 mg of aminoacetic acid and 85 mg of sodium chloride. For intravenous administration the dried product is disolved in 10 ml of water for injection.

SUMMARY OF THE INVENTION

I have discovered an unmodified intravenously injectable immune serum globulin having an ionic strength and a pH such that the monomer content of the immune serum globulin is greater than about 90% and the actual and latent anticomplement activity is maintained such that the immune serum globulin is intravenously administrable to a broad spectrum of patients.

The product of my invention is prepared by a method wherein an immune serum globulin (ISG) is solubilized to yield a solution of a certain protein concentration. The pH of this solution is adjusted, and the ionic strength of the solution is reduced, to a level such that the monomer content of the ISG is greater than about 90% and the actual and latent anticomplement activity is such that the ISG product is rendered intravenously injectable. The pH and ionic strength are maintained at the above levels during protein concentration adjustment, sterilization, filling into final containers, and the like.

One advantage of the ISG of the invention is that it is intravenously injectable thus avoiding the problems associated with intramuscularly injected material. Furthermore, the present product is substantially free from chemical modification such as occurs in reduction-alkylation, beta-propiolactone treatment, and the like.

An important feature of the product of the invention is that it is substantially free of actual and latent anticomplement activity and also substantially free of polymeric material or "aggregates". Particularly, the product of the invention exhibits enhanced stability over prior art preparations. The material may be kept at room temperature for long periods in the absence of additives with retention of its monomer content and lack of actual and latent anticomplement activity.

Another advantage of the invention is that the intravenously injectable ISG is virtually unchanged in physical measurements and biological functions. Thus, the antibody titers in the present material are not significantly different from the starting material.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The starting material for the process of this invention is unmodified human immune serum globulin. In the specification and claims the term "immune serum globulin" is used to define the substance also referred to in the literature variously as gamma globulin, IgG and immunoglobulin G. It consists predominantly and preferably of at least about 85 percent of the 7S species of gamma globulin, which has a molecular weight of about 160,000. Any remainder is preferably 9S species, with a molecular weight of about 300,000. Both standard immune and hyperimmune serum globulins, e.g., tetanus, rabies and hepatitis immune serum globulins, can be employed, the modified product being immune and hyperimmune ISG, respectively. Thus, a suitable starting material for the process of this invention is Cohn's Fraction II or Fraction III filtrate, See Cohn et at., *J. Am. Chem. Soc.* 68, 459 (1946); Oncley et al., ibid., 71, 541 (1949).

Fraction II, by ultracentrifugation studies, is predominantly (about 85 percent) the 7S (sedimentation constant of 7) species of gamma globulin with an average molecular weight of 160,000. The remaining protein is essentially 9S material with a M. W. of about 300,000. Wet Fraction II paste (approximately 30 percent solids) is commonly lyophilized to obtain dry ISG powder which is then dissolved and prepared for intramuscular injection as a 16.5 percent sterile solution. Either the wet Fraction II paste or the dry ISG powder is a suitable starting material for the process of this invention.

Gamma globulin obtained by any process which has essentially the same composition of protein components as found in the Cohn Fraction II or Fraction III filtrate can be used as starting material in the present process.

Both standard immune serum globulin and hyperimmune serum globulin can be employed as starting materials. As is well known, the latter is produced from plasma or serum obtained from selected donors who have much higher titers for a specific antibody than is normally found in the average population. These donors have either been recently immunized with a particular vaccine or else they have recently recovered from an infection or disease. These high titer sera or plasmas are pooled and subjected to the usual Cohn fractionation procedures up to the point of isolating Fraction II. The Bureau of Biologics (BoB) antibody standards for hyperimmune serum globulins presently are based on products to be given intramuscularly. These standards are based on the assumption a standard intramuscular dose of the reconstituted globulin (1–10 ml) will be administered. Because the amount of antibody required to achieve a desired immunological response is substantially less when administered intravenously, it will be apparent the I.V. dose will be substantially less than the I.M. dose which will produce the same serum antibody titer. Thus, the dose of intramuscular ISG and hyperimmune serum globulin must be higher than that required to achieve the same serum antibody titer when globulin of the same antibody activity is administered intravenously.

The starting wet paste or lyophilized powder is dissolved in a volume of water or other physiologically-acceptable carrier to provide a protein solution of a concentration of about 0.5–20% preferably about 5 percent. If Fraction III filtrate is employed, the aqueous solution must be concentrated by conventional techniques to the desired protein concentration. Any protein concentration may be used in this method; however, the above-recited range is preferred from a practical standpoint.

After the protein has been dissolved or concentrated, the solution is adjusted to a pH of about 3.5 to 5.0 preferably about 3.8 to 4.2, by addition of a physiologically-acceptable acid such as hydrochloric acid. In general, the pH is adjusted to a point whereat the monomeric material in the protein solution is maintained at a maximum. However, the pH must not be so low as to result in gelation. The temperature should not be harmful to the ISG material. Good results are obtained within the temperature range of about 0°–20° C. It is not necessary to hold the so-adjusted material for any period of time prior to the next step; however, the material may be held, if desired, without detrimental effects.

Following pH adjustment the protein solution is treated to reduce its ionic strength to a level at which the monomer content of the ISG preparation is greater than about 90%, preferably greater than about 95%, and more preferably greater than about 98%, and the actual and latent anticomplement activity is such that the ISG preparation is intravenously injectable. For this purpose the actual anticomplement activity should be greater than about 2 mg protein/C'H50 unit. The non-specific complement binding capacity of the product is determined using optionally titered complement and hemolysin. The complement binding capacity, known as anticomplement activity, is reported as mg protein product capable of inactivating (binding) one C'H50 unit. One C'H50 unit is defined as the amount of protein capable of inactivating 50% of complement in an optionally titered complement and hemolysin system.

The ionic strength ($\Gamma/2$) of the solution should be such that the product as a 5% protein solution has a nephelometric reading less than about 15 NTU (National Turbidity Units), preferably less than about 2 NTU. The ionic strength ($\Gamma/2$) is defined as follows:

$$\Gamma/2 = \frac{\Sigma\{[C^+]^2(Z^+)^2 + [C^-]^2(Z^-)^2\}}{2}$$

where

C^+ = cations including metal ions such as Na^+, K^+, Ca^{+2}, Mg^{+2}, and the like,

C^- = anions including halide ions such as Cl^-, Br^-, carboxylic acid salt ions such as acetate or citrate ions, and the like,

Z^+ = the charge of C^+, and

Z^- = the charge of C^-.

Preferably, the ionic strength, as defined, is less than about 0.001. The above treatment may be effected by standard procedures such as ultrafiltration, diafiltration, dialysis, etc., or combinations thereof. For example, the protein solution at the appropriate pH may be diafiltered with at least five volume exchanges of water, usually about 4–8 volume exchanges, to reduce the ionic strength to at least about 0.001. During this treatment the concentration of peptides and other impurities such as alcohol are also reduced, generally to trace amounts.

After or during the above treatment, the pH is measured and maintained within the range of about 3.5–5.0.

The protein concentration of the so-treated material is next adjusted to the level desired in the final product, such as, for example, 5%, 10%, 15%, and so forth. This adjustment is accomplished by conventional techniques

not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Again, the pH of the preparation is maintained within the range of about 3.5–5.0, preferably about 3.8–4.2.

Next, the ISG preparation is treated to render it tonic, i.e., to render it compatible with physiological conditions or render it physiologically acceptable upon injection. In this respect it is important to note that tonicity must be obtained without raising the ionic strength (as defined above) of the preparation. This end is achieved by adding to the ISG preparation an amount of an amino acid, such as glycine and the like, or a carbohydrate, such as maltose, dextrose, fructose, and the like, or a sugar alcohol such as mannitol, sorbitol, etc., or mixtures thereof sufficient to achieve tonicity. Thus, for example the ISG preparation may be mixed with about 10% maltose (on a weight to volume basis) to render the preparation tonic.

After the above adjustment the product is sterilized, usually be sterile filtration through appropriate media, and then filled into final containers. It is also possible to lyophilize the sterile ISG product after filling into final containers. For I.V. use the lyophilized material is dissolved in medically-acceptable water prior to injection. If the product has not been made tonic prior to lyophilization, the lyophilized material must be dissolved in a diluent containing medically-acceptable water and one of the aforementioned substances in an amount to render the preparation tonic.

The ISG of this invention is primarily intended for intravenous administration although the ISG preparation may also be administered intramuscularly if it contains the appropriate excipients. The composition aspect of this invention therefore relates to pharmaceutical compositions comprising a solution, in a pharmaceutically acceptable aqueous carrier adapted for intravenous administration, of an intravenously injectable ISG of this invention. The ISG is substantially pure. The ISG is present in these solutions in any concentration, either suitable for immediate I.V. administration or after dilution, e.g., with water or diluent as mentioned above, to acceptable levels, e.g., about 1–18 percent solution, preferably about 1–15 percent and more preferably about 10 percent for immediate administration, and about 16 percent for dilution prior to administration. The ISG can be administered intravenously alone or in combination with or in conjunction with other blood products, e.g., whole blood, plasma, Plasma Protein Fraction, fibrinogen, clotting factors such as Factor VIII, Factor IX concentrate, and so forth, and albumin.

In its method of use aspect, this invention relates to the intravenous administration, usually to humans, of a pharmaceutical composition as defined above. The composition is administered in a conventional manner, e.g., in an amount which provides adequate therapeutic amounts of antibody. For a 16.5 percent protein solution, about 1–25 ml is the customary single dose. Administration of subsequent dosages is usually within 1–3 weeks, depending upon the severity of the illness and the time of exposure thereto.

As mentioned above the products of the invention may be incorporated into pharmaceutical preparations, which may be used for therapeutic purposes. However, the term "pharmaceutical preparation" is intended in a broader sense herein to include preparations containing a composition in accordance with this invention used not only for therapeutic purposes, but also for diagnostic and reagent purposes as known in the art; for tissue culture wherein organisms such as viruses for the production of vaccines, interferon, and the like, are grown on plasma or on plasma fractions, e.g., Cohn Effluent II+III, Cohn Fraction IV, Cohn Fraction V, and so forth; etc. The pharmaceutical preparation intended for therapeutic use should contain a therapeutic amount of the present composition, i.e., that amount necessary for preventative or curative health measures. If the pharmaceutical preparation is to be employed as a diagnostic or a reagent, then it should contain diagnostic or reagent amounts of such composition. Similarly, when used in tissue culture or a culture medium the medium should contain an amount of such composition sufficient to obtain the desired growth.

The gamma globulin of this invention is substantially free from anticomplement activity, both immediate and latent.

Antibody titer is not significantly different from the starting unmodified gamma globulin, i.e., it is normal or hyperimmune, e.g., tetanus or rabies hyperimmune globulin, depending on the antibody titer of the starting ISG. The antibody molecules are bivalent, as indicated by their ability to precipitate with antigen.

Another characterizing feature of the ISG of this invention is its absence of proteolytic activity. It is known that some samples of ISG form fragments when stored. Such fragmentation is due to proteolytic digestion by a contaminating enzyme often presumed to be plasmin. Fragmentation is undesirable since it causes a decrease in the amount of active antibody in solution. The process of this invention sharply decreases the proteolytic activity in ISG to undetectable levels or at most to trace levels.

A primary and important characteristic of the present product is its stability. The product may be stored for extended periods of time without significant, if any, change in its antibody activity, monomer content, clarity, lack of anticomplement activity and so forth. For example, sterile, final container material prepared in accordance with this invention has been stored at room temperature on the shelf for greater than 6 months without significant changes in the above-mentioned qualities. This stability is obtained through pH and ionic strength adjustments as described above. The art heretofore has not recognized the relationship between pH and ionic strength on the one hand and intravenous injection on the other. As mentioned above, treatment of gamma globulin at pH 4 is known. However, the so-treated material was then returned to about pH 7 for administration to patients. Furthermore, addition of salts such as sodium chloride was employed to obtain tonicity.

A related benefit of the product of the present invention is its lack of buffer capacity. The present product is surprisingly administrable at pH 3.5–5.0. However, since the ionic strength has been reduced to a very low level, there is very little disruption, if any, of the physiological pH such as that which would occur with the administration of a material essentially buffered at pH 3.5–5.0 by the presence of salts.

EXAMPLES

The invention is demonstrated further by the following illustrative examples.

EXAMPLE I

The pH of Fraction III filtrate (2100 l.) from the Cohn fractionation scheme (Cohn et al, supra) was